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Cortical Responses to Modulated Light in the Human Subject

By

A. KAMP, C. W. SEM-JACOBSEN, W. STORM VAN LEEUWEN and L. H. VAN DER TWEEL

Received 31 March 1959

Abstract

KAMP, A., C. W. SEM-JACOBSEN, W. STORM VAN LEEUWEN and L. H. VAN DER TWEEL. *Cortical responses to modulated light in the human subject.* Acta physiol. scand. 1960. *48.* 1—12. — Electrical responses to modulated light have been recorded from a patient with depth electrodes implanted in the visual cortex. Low modulation percentages still gave a response even in the second harmonic of the stimulating frequency. The high frequency part of the frequency response curve showed a decrease corresponding to perception. Towards low frequencies also a steep decrease in sensitivity has been found. The responses in different places near to each other were not highly correlated. A discussion about the theoretical aspects is given.

The cortical responses to single as well as to repetitive flashes have been studied in animal and man. Although our method presents a different approach we refer to some of the publications relevant with respect to our own work. For a more detailed review we suggest that of BRAZIER (1953). Important data for the fundamental processes in electrophysiology of the eye can further be found in GRANIT's monograph (1955). The complicated response to single flashes recorded from the intact skull changes into a more regular pattern at higher flash rates. BARTLEY (1936) suggests that the high frequency response is reached by activation of separate elements each responding at a lower frequency but together presenting the original frequency. GREY WALTER (1953)

showed that in different parts of the brain, the phase, amplitude and also the frequency of the responses are depending on the recorded region.

MONNIER (1952) recorded the cortical responses in man, also in relation with the ERG. The negative C wave with a latency of about 100 msec is sometimes the only part of the response that is visible. BRAZIER (1957) followed the course of the response in the visual pathways with an averaging technique, and could discriminate in the different parts between primary and secondary (late) response. The important work was restricted, however, to single flashes.

Recently VAN HOF (1958) with numerical integration found a continuous change of pattern with increasing frequency resulting at high frequencies in a regular wave form.

Although these results are of great interest for certain aspects of cortical functioning, they are only partly correlated to the fundamental problems of vision. Most experiments have been performed in a more or less complete dark adaptation and the flashes used mostly were of high intensity. When repetitive flashing is used, the light adaptation is dependent on the duration of the stimulation, reaching its equilibrium only after some minutes. When the frequency is changed the light adaptation also changes if the energy per flash (as is commonly the case with electronic stroboscopes) remains more or less the same. With frequencies up to 10 cps the flashes certainly are not processed by the system in a linear way and are by no means comparable with the stimuli in normal conditions where successive and simultaneous contrasts are within reasonable limits. In Fourier analysis repetitive flashing is equivalent to 200 % modulation depth at the presented frequency and its harmonics, *i. e.* the peak values of the sinuses are two times the average intensity, such that even virtually negative light is present. Only at high frequencies where this modulation is decreased at an early stage in the visual process the normal state is approached. In that case, however, at constant light adaptation no variation of the modulation depth, nor of the frequency, is possible.

It is the merit of DE LANGE (1957, 1958) to have introduced a new method of photic stimulation for the investigation of CFF *e. g.* stimulation with modulated light. With this form of stimulation the light intensity varies sinusoidally around a certain value. Increasing the sinusoidal variation *e. g.* the modulation depth increases the difference between maximal and minimal light. In this way the strength of stimulation is enhanced without changing the average level which determines the light adaptation. In man the perception threshold under favourable conditions may be as low as 0.5 % modulation depth at a frequency of about 10 cps. At higher frequencies the modulation depth has to be increased to obtain a subjective response. When the measurements are carried out with a large visual field as was the case in our experiments curves are obtained as reproduced in Fig. 1, curve A (VAN DER TWEEL and DENIER VAN DER GON 1959). As DE LANGE has carried out his experiments with subjective determinations of thresholds only, it seemed of importance investigating

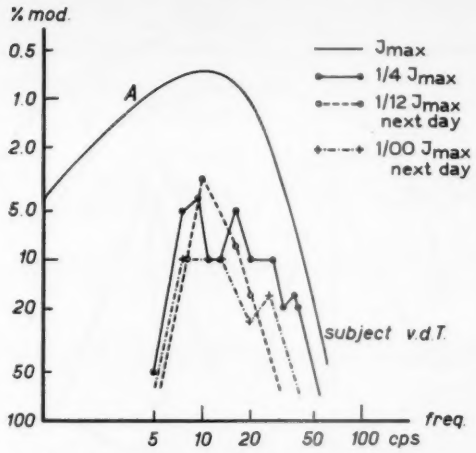

Fig. 1. Threshold modulation depth as a function of frequency (De Lange curves) with subjective determination and on base of electrophysiological responses with depth-electrodes. Although the electrophysiological curves have a higher modulation percentage, which can be ascribed to the disturbing influence of the spontaneous activity, the high frequency part is comparable to that in subjective vision, whereas the low frequency part is totally different.

also the electrophysiological responses to this form of photic stimulation. In this paper investigations are reported on the occipital responses in man. This has been made possible by the collaboration of a patient in whom depth electrodes had been implanted for other reasons by one of us (Dr C. W. SEM-JACOBSEN). An important advantage was that the recording could be made from comparatively small regions in the cortex, with small disturbances from movement artefacts. The results obtained in this way have been compared with data obtained by subjective methods. These latter experiments are tiresome and intelligent cooperation of the test subject is necessary. Therefore these experiments could not be performed in the patient and one of our own curves is given for comparison.

Instrumental technique

Light source

A television projection tube (DENIER VAN DER GON, STRACKEE and VAN DER TWEEL 1958) is used as a source for the modulated light. By means of two fast time bases an illuminated square is produced on the tube. The frequency of the time bases is well above CFF so that unwilled flicker effects do not occur and the light is very stable. The light intensity is controlled by comparing an electrical signal and the output of a photocell. In this way all kind of wave forms can be reproduced in the intensity. In the experiments to be described sinusoidally modulated light was used in most instances; to prevent long sessions only modulation percentages of 50, 25, 10 and 5 %

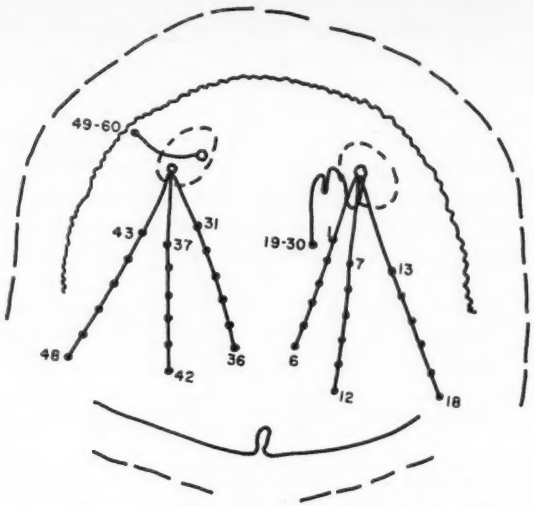

Fig. 2 A

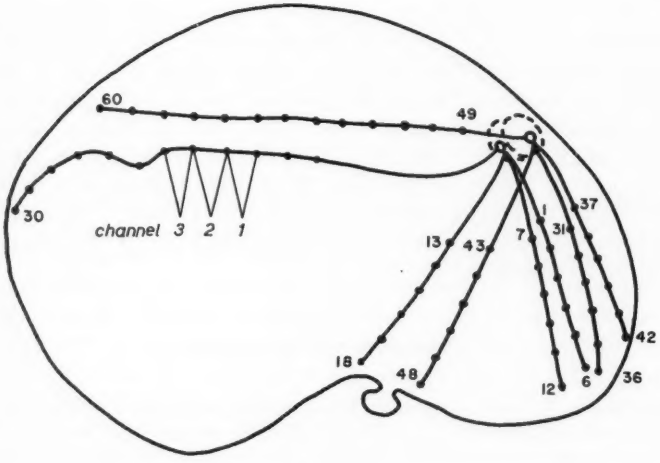

Fig. 2 B

Fig. 2 A and B. Electrode positions with indication of the channels from which the bipolar responses are produced. In all figures channels 1, 2 and 3 are the upper, middle and lower set of traces as indicated by the dashed lines.

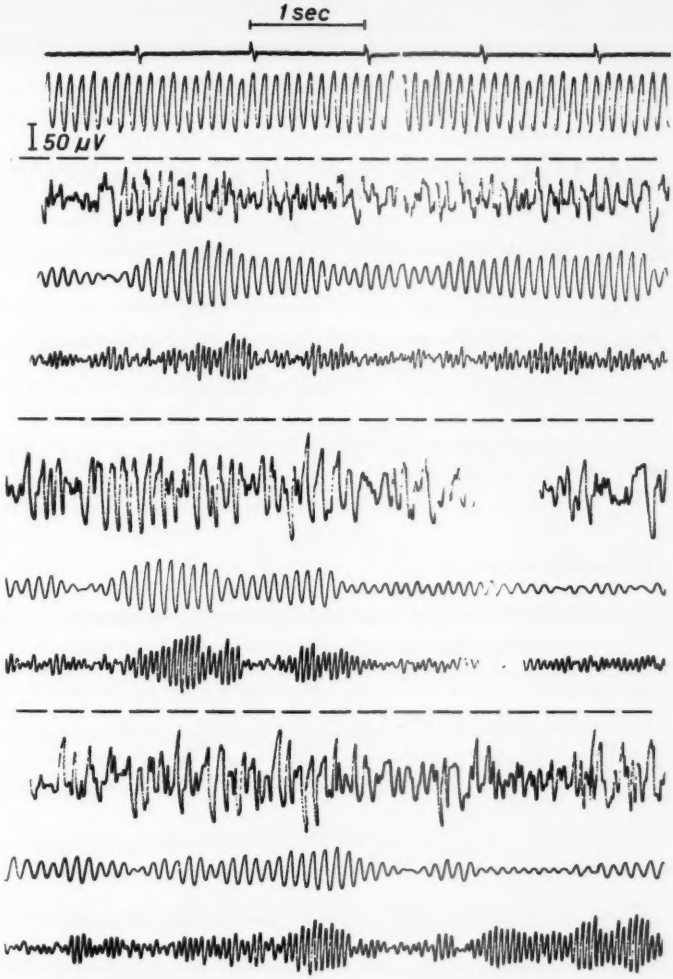

Fig. 3 A. Simultaneous recordings of three leads near to each other have been analysed when stimulated with a frequency of 10 cps and a modulation depth of 50 %. The analysis has been taken at 10 cps and 20 cps. Note the high fundamental response in the first channel occurring at another time than responses in the other channels. Second harmonic response is highest in the third channel although fundamental responses are low. The second trace represents the modulation signal.

have been used. In some cases square waves were presented with a light-dark ratio of 1/8 and 1/1. The average intensity was the same as with the sinusoidal modulation.

The light square was projected on both eyes of the patient and because of the geometry of the projection Schmit optique, the illuminated field as seen by the subject

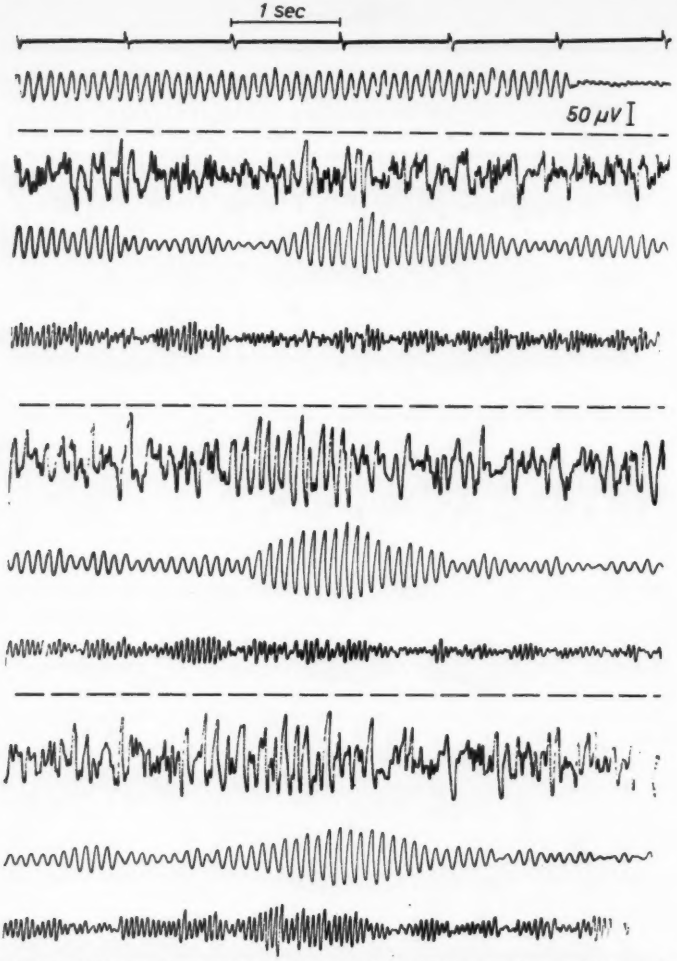

Fig. 3 B. The modulation depth is decreased to 25 %. A high second harmonic is still found in the third channel. Now the fundamental responses occur simultaneously. Owing to the tape recorder, the signals are somewhat distorted, as shown by the modulation signal, but this is of little influence.

was a ring with a diameter of 8° and a width of 1°. The maximal retinal illumination was about 10^5 trolands and the intensity could be easily decreased. The adaptation level in the room was always well below the brightness of the stimulating light source.

Methods

The signal is amplified and recorded with a conventional Grass electroencephalograph. The output of one channel is fed into a selective amplifier, which had been

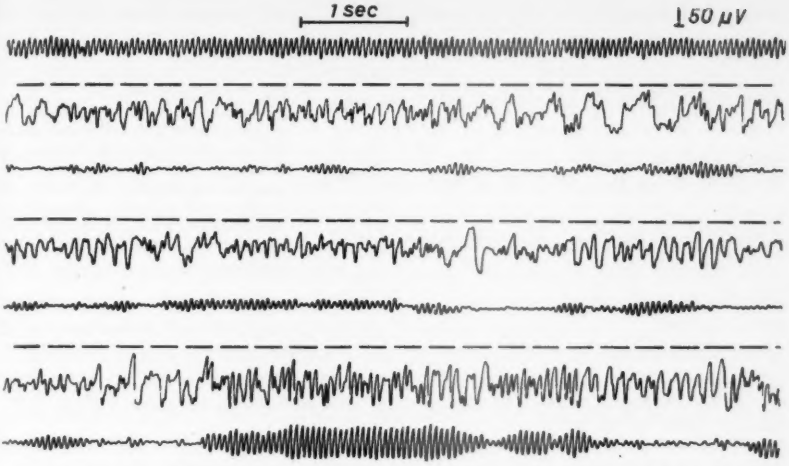

Fig. 3 C. The same channels have been analysed with 20 cps when modulated with 20 cps at 25 % modulation depth. Only the third channel shows a high response.

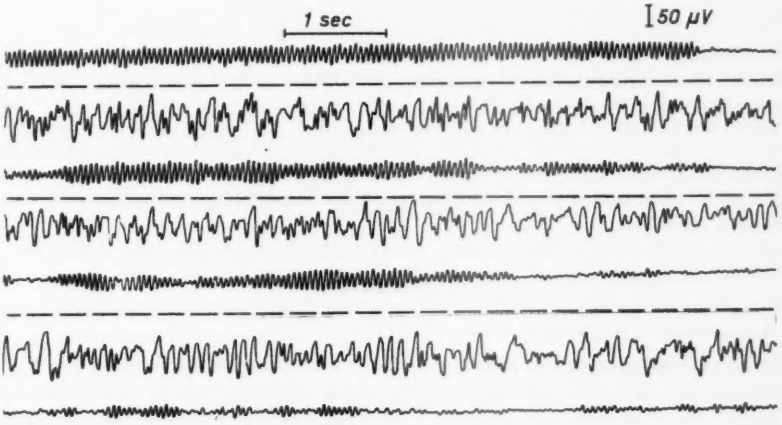

Fig. 3 D. As fig. 3 C, at another time during the same experiment, but now channels 1 and 2 show a response although not so high as channel 3 before.

tuned to the frequency of the audio oscillator used for the modulation. The output of the selective amplifier is recorded also with one EEG channel and generally a high selectivity ($Q = 20$) is used. The frequencies are adjustable in steps of 10 % starting at 2.2 cps. With another pen the light variations as "seen" by a photocell are recorded.

The three signals, as well as those of other channels, have been recorded also with a multichannel tape recorder allowing further analysis after the experiment.

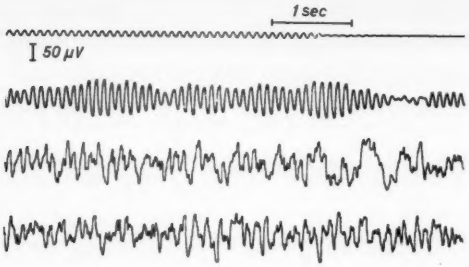

Fig. 4. A clear response is shown in channel 1 with a modulation depth of only 5 %. The modulation happened not to be decreased instantly. The amplitude of the analysed signal stays constant for a short time after the decrease in modulation depth. It is not improbable that this is still a real response.

Depth electrodes

The technique has been fully described by C. W. SEM-JACOBSEN *et al.* (1956). Fig. 2 shows the electrode positions determined by X-ray photographs. Signals have been recorded and analysed from various cortical and subcortical areas as well as from various areas of the scalp.

Determination of the responses

The determination whether or not a response has been present and if so, the magnitude of the response, is somewhat arbitrary owing to the following reasons. In all channels electric signals (to be considered as noise) non related to the stimulation are always present. These signals may have a variable contribution in the frequency band which is selected by the amplifier. Moreover the responses obtained in the human subjects are not constant. The amplitude of these responses changes in time and appears to be dependent on the attention of the subject. Generally the response was appreciated by the difference between the highest amplitude observed during stimulation and the highest amplitude during the periods of no stimulation. At threshold values it has sometimes been useful to compare the frequency given by the analyser and the modulation. If the phase differences remain constant during an appreciable time the response is considered to be caused by the stimulus even if the amplitude is not much higher than at zero signal.

Results

A. Responses to sinusoidally modulated light

The amplitudes of the response usually increase with increasing depth of modulation. It is not possible to deduct from this material an exact relation. Fig. 3 illustrates the interesting fact that in different channels the variations of amplitudes of the responses are not highly correlated. There is a good response in the second harmonic especially in channel 3. It is obvious that this harmonic response (at 20 cps) does not correspond strictly with the response at the flicker frequency of 10 cps in the same channel and does not correspond either with the harmonic responses in other channels. The responses in the same channel when the frequency of 20 cps itself is presented as a light stimulus show also the highest response in channel 3 which again is not correlated with that in the other channels. It seems probable that the cortical area in which

the fundamental response to a flicker frequency of 20 cps is highest is the same as where the second harmonic response (also 20 cps) to a flicker frequency of 10 cps is highest, even with low modulation percentages.

As has been stated above the determination of thresholds may be difficult, also because long sessions with the patient are undesirable. Nevertheless it has been possible to obtain frequency response curves which are reasonably reliable and reproducible at different sessions. In Fig. 1 the estimated modulation thresholds are plotted against the modulation frequency, also with different intensities. The curves show a distinct maximum at a frequency of 10 cps. The lowest modulation depth still producing responses is 5 %, perhaps even less as might be deduced from Fig. 4 in which the modulation by chance happened not to be drecreased to zero immediately.

At low frequencies the threshold rises rapidly and thus a steep decrease in sensitivity is seen. At 5 cps there is almost no response even with 50 % modulation. Also at higher frequencies the threshold rises but not so rapidly as at the lower side.

The intensity did not have a large influence in this regions of illumination which were always well above the photopic level.

B. Responses to pulsed light

As to be expected the responses to this form of photic stimulation are larger than those to the modulated light. At a repetition rate of 3.3 cps the responses are higher with a light-dark ratio of 1/1 than with 1/8 with the same average intensity. The second harmonic response was higher with a light-dark ratio 1/8 than with 1/1, although still present in the latter case.

Discussion

It has to be considered whether some of our results *e. g.* the form of the threshold frequency curves may be caused by the method and thus may be partially artefact. In the following discussion special attention has been given to this problem.

a) It has been discussed by Dr J. A. J. KLIJN in the February (1959) meeting of the Dutch society for physiology and pharmacology that the concept of threshold especially in the presence of noise has to be questioned. If a threshold is defined as a discontinuity in a stimulus-response curve the analysing method will not influence the measured value of the threshold in the absence of noise. It is still a point of discussion if threshold observations in subjective experiments on vision can be described in the mentioned way. In flicker experiments this is certainly unlikely.

As a measure for the behaviour of the system we have taken a response which was just distinguishable from the noise. Therefore the physical properties of the analysing system play an important role in the "threshold" value found.

We used a circuit with constant selectivity which means that the integration time is inversely proportional to the frequency.

Another criterion could be the modulation depth at which a constant response is reached for different frequencies. At present state of knowledge there is no preference for one criterion or the other. Because of the high slopes of the frequency response curves, we expect that our results are not much dependent on the choice of the criterion, so we have chosen the one which was most adapted to our instrumentation.

b) With our technique modulation of the stimulating light as low as 5 % could be detected in the cortical areas. Even lower percentages could be found in the geniculate body in cat (VAN DER TWEEL *et al.* 1958). Since the responses are recorded from bipolar leads, it is improbable that they are caused by far-away sources through electrical conduction. Also the responses in different channels from electrodes near to each other did not show the responses at the same time.

It is to be noted explicitly that a frequency analyser of the kind we used reacts on the harmonic content as given by Fourier analysis. Therefore very short signals especially those symmetrical around the electrical zeroline do not produce high amplitudes. Therefore the fact that small modulation percentages still are detectable with our method suggests that the responses are of a rather slow nature. It is interesting that this applies to the ERG, to the geniculate body as well as to the cortical responses. It seems improbable that the responses consisting of comparatively slow waves are conducted by the nervous pathways but they must be closely connected to the fundamental electrophysiological phenomena.

c) The highest sensitivity found with the electrophysiological method is at about 10 cps as is also the case with the subjective method. Moreover the sensitivity decreases in a similar way towards high frequencies in both methods. Since also in the geniculate body and even in the ERG similar reactions have been found, the original concept of DE LANGE that the attenuation at high frequencies occurs at an early state in the optical pathways gains in probability.

d) The sharp fall in sensitivity towards low frequencies has never been encountered in subjective measurements[1]. At frequencies of 4 cps the subjective sensitivity is still very high. In the geniculate body in cat where also with our method the low-frequency response was very low, action potentials could be obtained. These action potentials could be heard as a kind of noise, which at the low stimulation frequencies clearly had a rhythmic character. We suppose that this is caused by frequency modulation of the action potentials which probably represents the perception of flicker at these low frequencies. In one patient there was fast activity on presentation of light. With a high pass filter it was tried to select the action potentials. After rectification the signal was sent

[1] After the closing of this paper, KELLY (1959) gives curves in which also a sharp cut off for low frequencies occurs if the modulated area has no sharp boundaries.

through the analyser, but even with high modulation depth no response was found. It is possible, however, that the method was not sensitive enough.

There are two physical possibilities for an attenuation at low frequencies. In the first place as already mentioned the analyser reacts not well to signals of short duration. If the electrical response to modulated light were only a short impulse, lower frequencies would be attenuated approximately proportionally. There is no indication that the responses are of this kind, and moreover, the attenuation is much stronger.

In the second place with bipolar recording a travelling wave of a long duration will give a smaller amplitude, also approximately proportional to the frequency. We have also made recordings between one cortical electrode and the cortex and there is no indication that stimulation at low frequencies gives a better response in the latter case. Also, closely spaced electrodes do not give the same response at the same time, as referred before.

The sensitivity seems rather independent of the intensity. This is not surprising if we take into account the many nervous pathways and the fact that at the illumination levels and fields used the subjective relative threshold is also more or less independent of the intensity.

e) The concept of DE LANGE was that at flicker threshold the visual system was linearised. If a logarithmic retinal functioning is assumed, at a modulation depth of *e. g.* 10 % the second harmonic will be $^1/_{40}$ of the fundamental.

It is therefore highly important that even at low modulation percentages second harmonic responses were found. Moreover this second harmonic response was sometimes found without an equivalent fundamental response. If we do accept the approximation of linearisation in the case of modulated light a new physiological mechanism has to be supposed. Formalistically a second harmonic response could be caused by rectification. Physiologically we could suppose that increasing or — as well as — decreasing light gives a response which could be the generalisation of the on and off effect in the sense of adaptation and desadaptation. In the latter case and if more or less equal these two responses have to be represented in the same polarity to give the second harmonic response we found. That such hypothesis would be oversimplified is shown by the fact that at a frequency of 3.3 cps, with square pulses 1/1, we did not find a high response in the second harmonic.

f) The result of simultaneous analysis suggests that different frequencies might be dominating in different areas and also that the processes connected with CFF seem to be complicated.

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Venous Blood Flow Recorded with an Isothermal Flowmeter

By

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Abstract

MELLANDER, S. and R. F. RUSHMER. *Venous blood flow recorded with an isothermal flowmeter.* Acta physiol. scand. 1960. *48.* 13—19. — An isothermal flowmeter has been developed for recording of steady blood flow such as occurs in veins. The flow recording element consists of a fine heating coil enclosing a thermistor mounted on the tip of a catheter. The sensing element is held in the center of a vessel by five fine spring wires which also hold the dimensions of the vessel at a known constant size during recording. Heat is dissipated from the coil by blood flowing past the heating element. If the temperature of the thermistor is maintained at a constant level slightly above the blood temperature, the heat production in the heating coil has been found to be linear with blood flow over the range of flow met with in large blood vessels. The maximum error was found to be $\pm$ 5 per cent of full scale deflection. Changes in venous flow can be measured at a single site or the catheter can be advanced along venous channels, sampling blood flow at various positions. The distribution of the blood flow into the inferior caval vein from various vascular beds has been investigated in dogs. Refinements in the design are being performed to make the isothermal flowmeter suitable to clinical studies, if possible.

The catheterization technique has made possible blood sampling and pressure measurements in animals and human beings without interfering too much with the cardiovascular dynamics. Cardiac output can be calculated according to the Fick principle and the dye dilution technique, but these

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methods are not suited to continuous flow measurements. Numerous flowmeters have been designed for the measurement of regional blood flow in the anaesthetized animal, but the demand for a simple and reliable flow measuring device for clinical application is still not satisfied. New techniques are therefore required to record continuously regional blood flow in the intact organism. For some clinical purposes even qualitative information about flow would be of great interest.

In order to cover the need for flow measurements in intact animals and in human beings a catheter-flowmeter has been developed. This instrument is based on the thermal principle. An indirectly heated thermistor is exposed to the blood stream and the energy required to keep the element at a constant temperature, slightly above the temperature of the surrounding blood, is calibrated in terms of flow. This approach is somewhat related to classical thermostromuhrs (*e. g.* REIN 1928, BALDES and HERRICK 1937, BENNETT, SWEET and BASSETT 1944) and to a thermistor flowmeter previously described (FELIX and GROLL 1953), although some of the more important disadvantages of these devices have been reduced or excluded in the present design. In its simplest form, the isothermal flowmeter can be used for qualitative measurements of flow, but it can also be designed to provide quantitative data. It has been tested in model experiments but also applied in some experiments on dogs, where the distribution of the venous return from various vascular beds into the inferior vena cava has been recorded. A preliminary report on this flowmeter has previously been presented (MELLANDER 1958).

Method

1. Design principles

The flow-sensing element consists of a tiny thermistor around which has been wound a very fine wire.[1] Flow is recorded electrically. The coil of wire forms a heater and the thermistor serves as a sensitive heat-detecting device. If the element is positioned in a stream of blood, heat is dissipated from the heating coil at a rate which is proportional to the flow of the surrounding fluid. To maintain the temperature of the element at a constant level, slightly above the temperature of the surrounding fluid, heat must be produced in the coil at the same rate as heat is transferred to the blood. The rate of heat production, expressed as power ($P = E^2/R$), applied to the coil is calibrated in terms of flow and recorded continuously on a suitable chart recorder.[2] The maintenance of a constant temperature of the element is accomplished by adjusting automatically the voltage applied to the heating coil. A circuit diagram showing the electronic arrangement is illustrated in Fig. 1. The rate of heat production in the coil is found to be linearly related to flow over a wide range. (Fig. 2. Power is here simply expressed as $(\text{voltage})^2$ as the resistance of the heating coil, R, is approximately constant.)

[1] Thermistor: Type 33A3, Size: 2 × 0.5 mm.
Source: Victory Engineering Corp., Union, N. J., USA.

[2] P = Power, E = voltage applied to the heating coil, R = resistance of the heating coil.

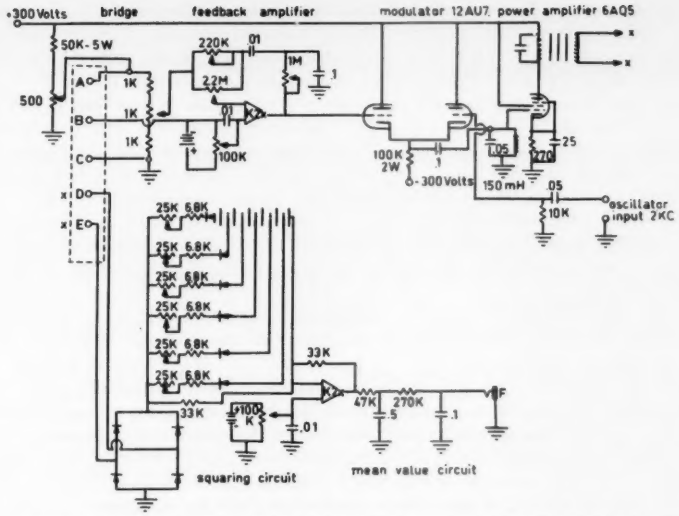

Fig. 1. Schematic circuit diagram of the isothermal flowmeter. Sensing thermistor connected to A—B, reference thermistor to B—C and heating coil to D—E. A—E connected to recorder. K2x indicates a high-power plug-in amplifier (G. A. Philbrick Researches, Inc., Mass., USA).

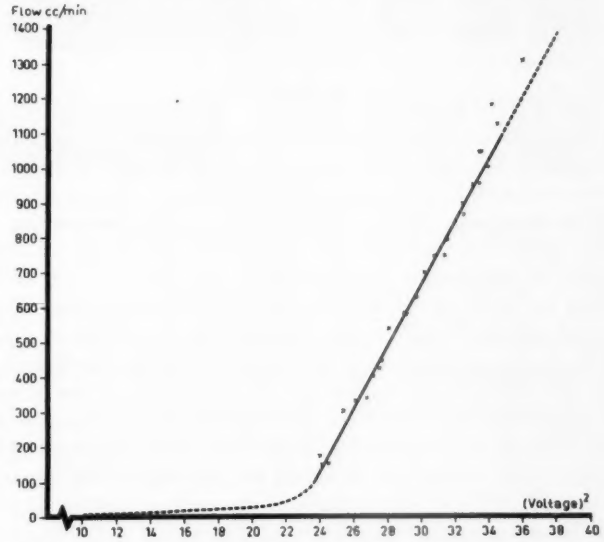

Fig. 2. Calibration curve of the isothermal flowmeter in model experiments with blood flow in tubing of 10 mm internal diameter, showing the linear range for flows from approximately 100 to 1,100 ml per min.

In practical use the flow-recording element is mounted on the tip of a cardiac catheter with a reference thermistor upstream from it for compensation of spontaneous temperature changes of the blood (Fig. 3). During insertion of the catheter the sensing element is enclosed by five flexible springs aligned along the axis as shown in Fig. 3 A. For recording flow, the springs are expanded by pulling out a calibrated center stylus at the outer end of the catheter (Fig. 3 B). The sensing element is held at a fixed position in the center of the vessel by the five springs, which also hold the lumen of the vessel at a known constant size during recording. The construction of this part of the device met with considerable practical difficulties, as some details must be very delicate in order to keep within the dimensions of an ordinary heart catheter. The spring arrangement is, however, necessary in order to get quantitative information about blood flow as the flow characteristics will vary with the bore of the vessels and the flow profile.

2. Mode of testing

In well controlled experiments the maximum error was found to be $\pm$ 5 per cent of full scale deflection. The frequency response was about 5 cps. The baseline drift over one hour of constant flow was less than 2 per cent of full scale deflection.

Model experiments with the isothermal flowmeter were performed with flow of water or blood in tubes of varying diameters. Beside the model tests, experiments were performed on nine anaesthetized and heparinized dogs in a study designed to show the distribution of outflow from various vascular beds into the inferior vena cava. The flowmeter was then inserted into the right external jugular vein and advanced to a site in the inferior vena cava about three centimeters above the diaphragm. As illustrated in figure 4 D, fine nylon loops were passed around the major vessels at their entrance into the vena cava, so that the veins could be briefly and completely occluded without interfering with flow from other vessels. This arrangement permitted the estimation of blood flow from separate vascular beds in terms of decreased caval flow. Direct calibration *in vivo* was performed with a graduate and a stop-watch via a T-tube inserted into the inferior caval vein just proximal to the gauge.

Results

Figure 4 summarizes the results from a representative experiment. The first tracing (4 A) shows the flow pattern in the abdominal vena cava with a cardiac cycle and a respiratory cycle indicated. Below is shown a record taken at slow paper speed with the flow meter placed in the same position. Fig. 4 B illustrates a calibration curve *in vivo*. In the first part of Fig. 4 C the circulation through the left kidney was obstructed (*a*), then in both kidneys (*b*). In *c* the flow in the regions drained by the left and right common iliac veins was occluded and in *d* also the flow through the two kidneys. Lastly, in *e* the renal blood flow together with the outflow from the portal vein was occluded. In such experiments an average of 23 per cent of the blood flow in the inferior caval vein was found to be derived from the two common iliac veins, 27 per cent from the two renal veins, 26 per cent from the portal vein and 24 per cent from other tributaries, including the drainage of the hepatic artery, the lumbar veins, the spermatic veins and the deep circumflex iliac veins, as well as a few other minor vessels.

The comparatively small renal flow was somewhat surprising since it is generally believed to comprise roughly 25 per cent of the total cardiac output.

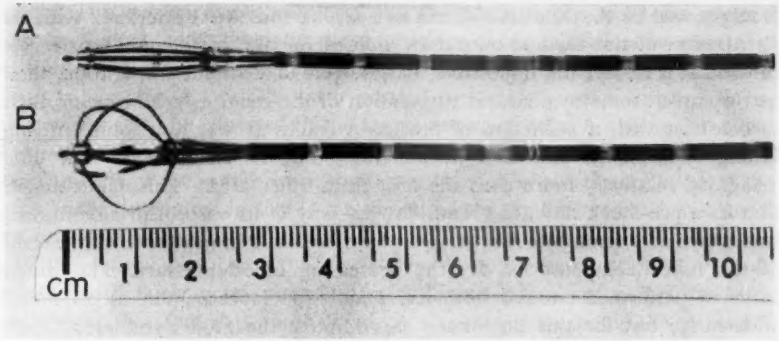

Fig. 3. Isothermal catheter-flowmeter with the sensing element mounted in the center of five flexible springs and the reference element placed at the tip of the catheter. For insertion of the flowmeter into a vein the springs are aligned along the axis (upper figure). For recording blood flow the springs are expanded by pulling out a calibrated center stylus at the outer end of the catheter so as to fix the flowmeter in the desired position and to keep the lumen of the vessel at a known constant size during recording (lower figure).

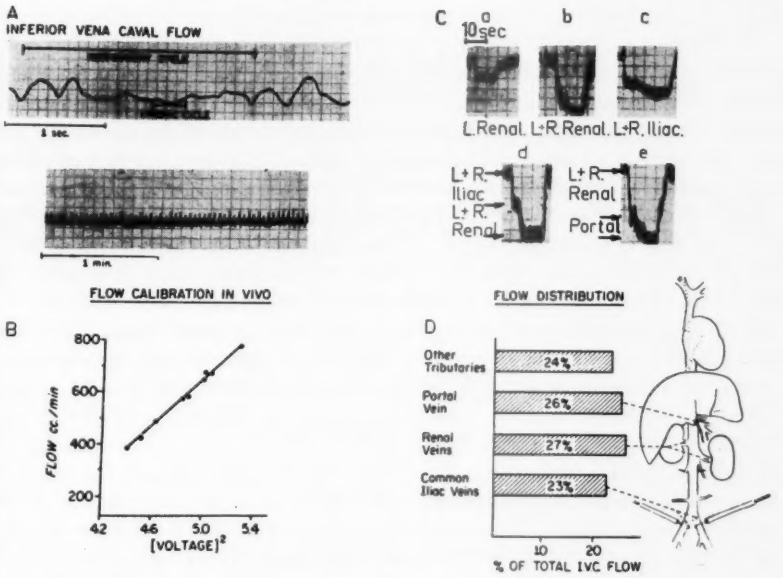

Fig. 4. *A.* Flow records on Sanborn polyviso recorder with the flowmeter installed in the abdominal vena cava. *B.* Flow calibration *in vivo*. *C.* Tracings showing the decrease of blood flow in the thoracic vena cava during separate occlusion of flow in different vascular beds as indicated. *D.* Diagram of blood flow distribution in the inferior vena cava. For further explanation see text.

It might well be that the anaesthesia as such has somewhat interfered with the distribution of the cardiac output by influencing the cardiovascular nervous control. It is further not impossible that, in spite of a careful preparation, there had occurred some mechanical stimulation of the renal arteries causing them to constrict with a reduction of flow as a result. It was also found in long lasting experiments and after haemorrhage that the renal blood flow often decreased relatively more than the flow from other areas. This might suggest that in a pre-shock state, the renal flow by way of its vasoconstrictor fibres is relatively more strongly reduced than the blood flow in other areas as a result of the reflex compensation for the decreasing blood pressure. The studies hitherto performed should, however, mainly be looked upon as an *in vivo* wide-range test for this flowmeter, to complete the model studies.

Discussion

In their careful evaluation of the classical thermostromuhr, GREGG and co-workers (GREGG *et al.* 1942, SHIPLEY, GREGG and WAERN 1942) pointed out the many limitations of this method. The most important ones imply: 1) errors caused by pulsatile flow, retrograde flow and zero flow, 2) instability and calibration difficulties, 3) poor frequency response, 4) non-linearity.

A different approach allowing a more reliable, continuous blood flow recording in the intact organism has therefore been utilized so that the mentioned disadvantages could be avoided or eliminated. By using the isothermal flowmeter on the venous side instead of the arterial side of the circulation, as was generally the case with the classical thermostromuhrs, errors caused by retrograde flow and pulsatile flow were reduced. The results of *in vivo* experiments indicated that rhythmical fluctuations as they occur in the larger veins had little influence on the accuracy of the method. — As the isothermal flowmeter was designed for such veins where only relatively high flow rates are met with, the limitations depending on very low or zero flows need not be taken into consideration in this case. — The close physical contact between heater and heat-detecting device, the small size of the unit and the automatic control of a constant temperature of the element constitute the basis for a good frequency response with the isothermal flowmeter. Although this device was primarily designed for mean flow measurements, it seems quite possible, if desired, to build a unit with a frequency response adequate also for pulsatile flow. — An overall non-linear response curve was a feature common to all previous thermal flowmeters, which showed great sensitivity to flow changes in the low range and a rapidly decreasing sensitivity at larger flows. This drawback was closely related to the calibration problems of thermal flowmeters and made these methods extremely cumbersome, particularly so, since a calibration *in vivo* was frequently necessary. As shown in figure 2, the isothermal flowmeter has a linear range for flows from less than 100 cc/min. to 1,100

cc/min. This curve is based on model experiments in a tube of 10 mm internal diameter, which corresponds to the dimensions of the inferior vena cava of a medium sized dog. Similar calibration curves were also obtained in experiments *in vivo* (Fig. 4 C). The linear portion is extended well above and below the flow range to be expected in the larger vessels of dogs. A calibration curve obtained *in vivo* was reproducible in experiments on different animals, provided the diameters of the vessels were the same. Because of the distensibility of the veins, slight differences in size of the vessels where the flow recorder is placed, can be compensated for by graded expansion of the springs on the flowmeter until the desired diameter is obtained. Therefore, one calibration curve may be used with fairly good accuracy for different animals of about the same size. — As this device is constructed as a catheter-flowmeter the necessary operative procedure is limited to a venous puncture. The technique should therefore be no more complicated than a routine catheterization and it offers the possibility of direct flow measurements in unanaesthetized intact animals, normal subjects and patients. Some technical and practical details remain to be solved, however, before the arrangement of the springs of the flowmeter device at the catheter end, which allow the measurement of the vascular diameter, is stable and safe enough to justify its use in man. The springs must, for instance, necessarily be very delicate but also strong enough so that not one of them breaks so as to cause damage to the catheterized vein. Further development of such details being performed.

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Quantitative Measurements of Sweat Gland Activity Using the Ninhydrin Method

By

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Abstract

PONTÉN, B. *Quantitative measurements of sweat gland activity using the ninhydrin method.* Acta physiol. scand. 1960. *48.* 20—28. — When a piece of paper is pressed against a sweating surface of the skin, the sweat together with its content of dissolved amino acids is absorbed. The print on the paper forms a pattern which, after evaporation, is represented by the amino acids and other substances. By using the ninhydrin reaction these amino acids can be stained and the print on the paper visualized. The present paper reports on a modification for quantitative analysis. The results are discussed and compared with the findings of earlier authors as regards the onset of sweating, variations in its flow, and similarities and dissimilarities between different skin surfaces.

The physiology of sweat secretion has always been the subject of keen interest and studies on the function of the sweat glands have been carried out since the very early days, as is exemplified by KUNO (1956) in the introduction to his classical monograph "Human Perspiration". A very large number of methods of studying the glands have been published in the literature. Major reviews on the different methods have been published by BRUN 1954, ROBINSON and ROBINSON 1954 and LENTZER and BOUMAN 1957.

The Ninhydrin Method

The sweat contains a large number of amino acids: 10 according to HIER, CORNBLEET and BERGEIM (1946), and 14 to 16 according to Japanese authors (KUNO 1956). Their concentration is estimated differently by different authors, but probably lies between 1.1 and 10.2 mg per 100 ml (KUNO). However, individual variations are

wide. Furthermore, in one and the same person the level varies according to the rate of sweating, being high when perspiration is slight and low when it is profuse (ROTHMAN 1954, KUNO 1956).

In 1954, ODÉN and VON HOFSTEN described a new method of taking fingerprints on paper. It was based on the well-known ninhydrin reaction: the amino acids of the sweat were stained with ninhydrin. If a piece of paper is pressed against a sweating skin surface it absorbs the sweat together with the amino acids dissolved therein. After treatment with ninhydrin solution a coloured pattern corresponding to the sweat pattern on the skin surface emerges. With this technique ODÉN and VON HOFSTEN were also able to visualize fingerprints made many years earlier.

MOBERG (1958) who recorded objectively the loss of sudomotor function in peripheral nervous lesions of the hands was the first to grasp the significance of the method for clinical purposes, and he modified it for practical use. He carried out parallel tests with DOLE and THAYSEN's (1953) modification of RANDALL's (1946 a) technique and found a very close correspondence between the results of the two tests, despite their differing basic principles.

Present Investigation

Both MOBERG (1958) and MANNERFELT (1958) have used the ninhydrin reaction for qualitative analysis of sweating on skin surfaces affected with nervous lesions and on grafts. Owing to its precision and simplicity, the method lends itself to modification for quantitative analysis. In order eventually to utilize this method in its quantitative modification for study of the sweating of grafts, it was first tested by investigating the onset of sweating, the function of the sweat glands in different areas of the body, and the normal rhythmic variations in the outflow of sweat. Phenomena already closely studied earlier by KUNO (1956), RANDALL and his associates (1946 to 1958), and ALBERT and PALMES (1951) were selected for investigation. These workers used different methods but arrived at very much the same results.

Material and Technique

Paper: Most types of ordinary white writing paper can be used. The paper, however, must be dry and free from amino acids and other substances reacting positively to ninhydrin. Nor should the paper be too porous, for the patterns will then be blurred. It must be handled with utmost care to avoid interference from the examiner's own prints.

Stamp: A piece of foam-plastic 1.5 cm thick on a flat wooden tray with handles has been found to afford an even distribution of pressure over uneven skin surfaces.

Solutions: 1) 1 per cent ninhydrin solution in acetone. This is acidified before use with a few drops of glacial acetic acid per 10 ml ninhydrin solution.

2) For fixation: 1 per cent solution of copper nitrate in a 5: 95 mixture of water and methyl alcohol or acetone acidified by the addition of a few drops of concentrated nitric acid per 100 ml.

Amino acids are normally present even on nonsweating skin surfaces. These probably derive largely from excreted and evaporated sweat, but a small amount may be regarded as the side-products of the keratinization process (ROTHMAN 1954). The

skin area to be examined must, therefore, first be washed with alcohol or ether in order to obtain a distinct print of the individual sweat pores. This applies particularly to the regions in which mental sweating is continuous, such as the volar surfaces of the fingers. Fig. 1 a) shows the print of a thumb made without prior cleansing of the skin; Fig. 1 b) a print of the same surface 15 sec after washing with alcohol. If only a limited area is to be tested, the surrounding skin can be screened off with adhesive plaster. With the flat foam-plastic covered wooden tray a piece of paper is pressed firmly against the skin for roughly one second. Further prints are taken at the intervals desired, as after 5, 10, or 15 sec. The sweat is absorbed onto the paper which can be stored almost indefinitely and still give a clear print when visualized. For visualization the paper is dipped in the acidified ninhydrin solution and allowed to dry in the air. It is then warmed in a thermostat at 80 to 90° C for 5 min. A higher temperature can be used but may lead to slight brown discolouration of certain types of paper. A distinct print emerges, showing the sweat-pore orifices as small violet dots. The pattern may continue to "emerge" for a few hours, but usually it should be analysed immediately the paper is removed from the thermostat, or at least within 24 hours. This because the colour fades gradually so that the smallest and faintest dots may be lost. The small dots are readily counted with the help of a magnifying glass.

If the print is to be stored for a long period, it is dipped in copper nitrate solution and air-dried. It then turns pale red; the colour is stable but the pattern loses some of its clarity. Consequently, it was found best to count and photograph the dots on the original print, unless it was to be stored for any length of time. Some difficulty was encountered in photographing the prints as the violet colour was weak in contrast, but using an ordinary process-type film or plate no retouching is necessary to obtain sufficient depth of colour for the dots to stand out clearly against the background.

Comments on the technique: In each print there are a larger or smaller number of dots according to the rate of sweating. When slight, the few dots are small and somewhat stronger in colour. Profuse sweating for a long period produces paler colour owing to the lower amino-acid concentration. However, tests made after 90 min profuse sweating showed the concentration to be sufficiently high to give distinct prints.

In profuse sweating the sweat shows a tendency to flow away from the pores, but these can readily be distinguished from each other if prints are taken at sufficiently short intervals (Fig. 2 c). If a long period elapses between prints, the sweat flows out over several pores and counting becomes difficult or impossible. This may occur during maximal sweating in areas in which there are many sweat glands or in which copious amounts of sweat are produced even if prints are taken at very short intervals (Fig. 3 c).

During moderate sweating, an excessive delay between prints may lead to a great concentration of the sweat due to evaporation which in turn gives a strongly-coloured print (Fig. 4 c).

In most cases an interval of 15 sec between prints has been found suitable; during more profuse sweating recordings can be made every tenth or even every fifth second.

Each dot on the paper corresponds to a small drop of sweat on the skin. This drop marks a pore which is either producing sweat as the print is taken or has done so since the last print was taken. The pattern thus indicates the number of sweat ducts which are excreting or have excreted during the preceding 15-sec period. Some evaporation may have occurred during this period, but if the individual sweat drop has not evaporated completely it will emerge distinctly owing to the increase in amino-acid concentration. Only when evaporation is complete does the sweat pore fail to appear on the print, since no fluid is then absorbed by the paper. Complete evaporation may, of course, occur but it is a factor of minor importance during so short a period as 15 sec; this is illustrated by the correspondence between the present author's results and those of RANDALL who applied the pressure for 15 sec.

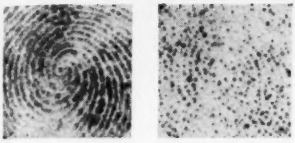

Fig. 1 a. Print of the thumb, volar surface. No preparation of the skin. The size of the original print in this as in the following cases was 1 square centimetre.
Fig. 1 b. The same surface 15 sec after washing with alcohol.

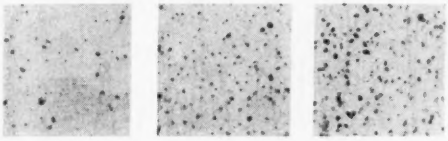

Fig. 2 a, b, c. Forearm, volar surface. Increasing rate of sweating.

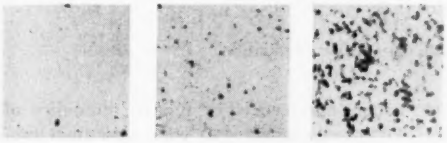

Fig. 3 a, b, c. Dorsum of the foot. Different rates of sweating. c) shows profuse sweating in which there is some difficulty in distinguishing between the individual sweat pores.

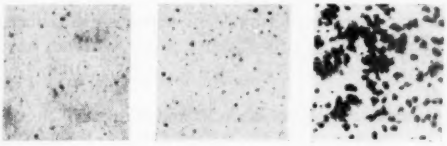

Fig. 4 a, b, c. Forehead. Slight to moderate sweating. c) print taken 5 min after b).

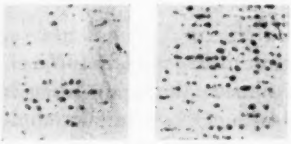

Fig. 5 a, b. Thenar region. Different degrees of sweating.

If a very large amount of sweat is produced by a gland, one print may not absorb all the fluid and the same dot will appear on the next print. The paper must, then, be capable of absorbing all the sweat produced during fairly profuse sweating. The paper used in the present investigation appears to have been sufficiently highly absorbent, since a blank print was obtained after a preceding one had shown a copious amount of sweat. During more profuse sweating, however, it seems likely that one and the same dot representing a single sweat pore may appear on more than one print.

Experimental Conditions

1. In order to study the rapid onset of sweating, the subject was placed in a bath which was quickly filled with water as hot as could be tolerated. The temperature of the water ranged from 46 to 48° C and the whole body except the head, arms, and feet were immersed.

2. For concurrent investigation of sweat outbursts and the function of the sweat glands on different areas of the body after milder thermogenic stimulation, the subject lay nude on a stretcher in a climatic chamber in which the air temperature could be

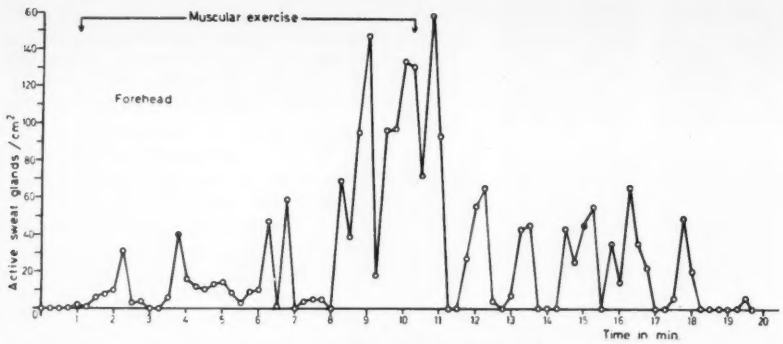

Fig. 6. Relatively slow onset of sweating after moderate stimulation.

accurately regulated and maintained at a constant level. The relative humidity of the air was between 35 and 55 per cent. Gymnastic movements were sometimes prescribed to increase the sweating.

3. Mental sweating was investigated in patients suffering from nervousness before an operation or the like. These tests afforded sufficiently distinct prints without any extra stimulus.

4. Only eccrine sweating was studied.

5. The subjects examined were healthy males aged between 20 and 35 years.

Results

Figs. 1 to 5 illustrate different rates of sweating on different body surfaces. The number of active sweat glands per square centimetre during sweating is dependent upon many factors: race, sex, age, individual, body surface, stimulus, and method of examination are among the more important. Consequently, comparison of the results of different series presents difficulty. However, the results arrived at with the ninhydrin method correspond closely with those obtained by other authors under similar conditions.

Fig. 6 shows a relatively slow onset and fairly moderate sweating which does not reach the maximum. The peaks in the curve become higher and broader with increasingly short intervals of inactivity, marking the increase in the number of glands in action and the rising volume of sweat excreted.

A rapid onset of sweating after sudden and extreme stimulation (hot bath) gives a curve of different configuration (Fig. 7). After a short time-lag a very large number of glands rapidly start to function and excrete sweat for long periods without a pause. Since this does not agree with the findings of RANDALL, HERTZMAN and EDERSTROM (1951), the experiment was repeated and prints taken at 5-sec intervals, but even then no periodicity was demonstrable at the onset of sweating. However, variations occur in the number of open pores once a certain maximum has been reached — but these fluctuations are less

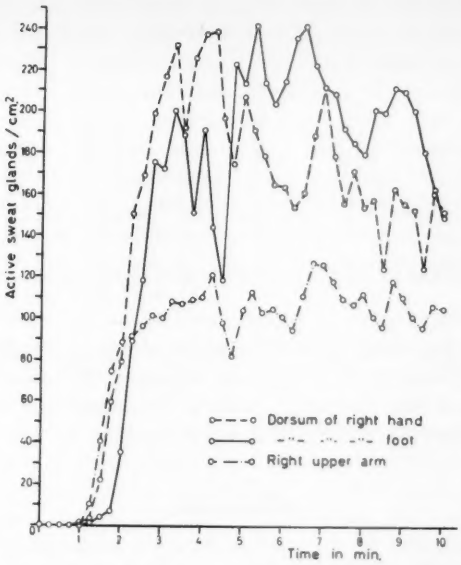

Fig. 7. Sudden onset of sweating after extreme thermogenic stimulation. Synchronous recordings on different parts of the body.

marked and lie at an appreciably higher level than during moderate sweating. Fig. 7 also shows that there is only a very slight time-lag, or none at all, between the onset of sweating in different areas of the body after this extreme stimulation. However, concurrent recordings on different surfaces of the body during moderate thermogenic stimulation reveal definite discrepancies in the time of onset of sweating (Fig. 8).

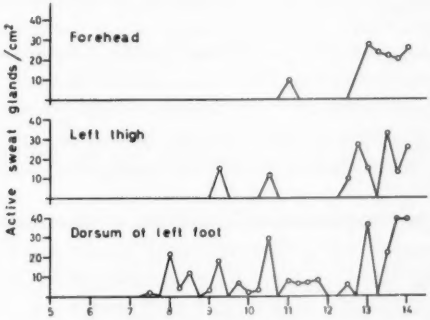

Fig. 8. Relatively slow onset of sweating. Synchronous recordings on different parts of the body.

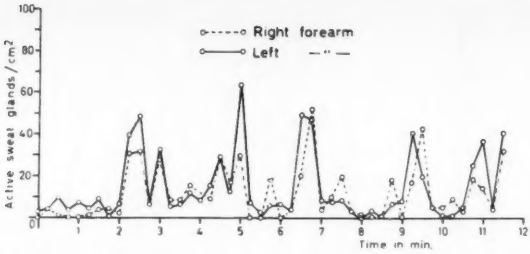

Fig. 9. Moderate sweating on symmetrical body surfaces. Synchronous recordings.

The curves in Fig. 7 also show the periodic waves to be fairly synchronous even when widely separated surfaces are compared. The sweat glands in skin areas close to each other are most closely synchronized, and the same applies in even higher degree to symmetrical skin areas (Fig. 9).

Discussion

The cyclic variations in the outflow from the sweat glands demonstrated in the present investigation have long been recognized. They have been studied and verified by several authors (RANDALL 1946 b, ALBERT and PALMES 1951, and KUNO 1956). Each individual gland produces a rhythmic flow and groups of glands alternate in flow, so that when some ducts close others take over. The studies of RANDALL, MCDONALD and STALZERS (1958) suggest that certain groups of glands in a given area act as a functional unit, probably owing to a common terminal innervation. As long as sweating is slight or moderate, then, definite pauses in the outflow of sweat are demonstrable and for short periods all the ducts are closed (Fig. 6). During heavy and profuse sweating rhythmic variations in the number of open pores can still be noted, but these are less marked and no complete stoppage in the outflow from all the sweat glands occurs (Fig. 7).

When sweating increases, the number of glands in action first rises and then the volume of sweat produced (RANDALL and MCCLURE 1949). This is reflected on the curves by higher and broader peaks. As mentioned earlier, I failed to demonstrate any such periodicity at the sudden onset of sweating. This was probably due to the suddenness and intensity of the thermogenic stimulus applied.

It is of interest to compare these two types of thermogenic sweating with the findings of LOBITZ and OSTERBERG (1947) on analysing mental sweating on the palm of the hand. These authors demonstrated the existence of two different types of sweating, intermittent and profuse, in which the function of the glands was in principle similar to that illustrated in Figs. 6 and 7.

There are two diverging views as to the regional distribution of the onset of sweating. KUNO (1956) holds that sweating starts simultaneously over the whole body surface. RANDALL's group and others observed time-lags on concurrent examination of different areas of the body (RANDALL, PEISS and RAWSON 1958, HERRMAN, PROSE and SULZBERGER 1952). The results of my investigation indicate that time-lags in the onset of sweating are dependent upon the intensity of the stimulus applied. Several tests with moderate stimulation corroborated RANDALL's findings, that the start of sweating follows the order foot, calf, thigh, trunk, upper extremity, face (Fig. 8), while an extreme thermogenic stimulus induced an almost simultaneous onset of sweating in different parts of the body (Fig. 7).

Concurrent recordings on different body surfaces give curves showing fairly close correspondence, probably the manifestation of common and simultaneous central impulses. This correspondence was also demonstrated by earlier authors (RANDALL 1946 b, ALBERT and PALMES 1951, and MCGREGOR 1952). The discrepancies between curves representing widely separated skin surfaces may possibly be due to morphologic dissimilarities between different sweat glands and/or to the variations in sweating rates in different parts of the body reported by many writers (LADELL 1945, WEINER 1945 and RANDALL and MCCLURE 1949).

The present investigation has shown the ninhydrin method to be both simple and reliable for analysis of the outflow from sweat glands under different conditions. Neither the paper nor the skin need preparation other than simple cleansing of the skin surface. In contrast to similar methods described earlier, prints can be taken at very short intervals since the paper need be applied to the skin for little more than a second. A point of practical value is that the print can be photographed and/or fixed for storage for long periods.

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Acta physiol. scand. 1960. 48. 29—35

From The Department of Clinical Physiology, University of Göteborg, Sweden

Carbon Dioxide Tension and pH of Lymph and Arterial Blood in Anaesthetized Dogs

By

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Abstract

CARLSTEN, A. and B. SÖDERHOLM. *Carbon dioxide tension and pH of lymph and arterial blood in anaesthetized dogs.* Acta physiol. scand. 1960. *48.* 29—35. — Thoracic duct lymph in the dog has a lower pH and a higher P_{CO_2} than arterial blood irrespective of the degree of ventilation, oxygenation and induced acidosis. The difference in P_{CO_2} between lymph and arterial blood is about 18 mm Hg. Such a high P_{CO_2} difference is taken as an indication that tissue CO_2 tensions have been underestimated with previous techniques.

Tissue tensions have been measured by introducing electrodes for P_{O_2} (DAVIES and BRINK 1942, KAY and COXON 1956, MONTGOMERY and HORWITZ 1950) into the tissues and by introducing gas pockets (CAMPBELL 1931, VAN LIEW 1956) into the subcutaneous tissue. Both methods are dependent upon the topographic relation to the capillaries and presumably give gas tension values close to those of venous blood. A closer approach to the tissue tensions is presumably via the lymphatics as lymph is a certain part of the extracellular fluid and thus derived from the immediate environment of the cells. A comparison of gas tension in lymph and blood with different forms of acidosis and alkalosis ought to provide us with more dynamic aspects of this relationship.

Blood gas tensions have been studied thoroughly but the same analyses of lymph are very scanty. A comparison between thoracic duct lymph and blood has been published by v. KRÜGER (1925) and GESELL (1929). According to KRÜGER lymph in man and dog is more alkaline than serum and plasma. GESELL, however, found that thoracic duct lymph as a rule was of a lower pH than arterial blood. HEIM and LEIGH (1935) reported data on P_{CO_2} and pH of cervical lymph. This lymph was more alkaline than jugular venous blood plasma.

The P_{CO_2} was calculated from the CO_2 content using Henderson-Hasselbalchs formula and assuming a pK' similar for lymph and blood plasma.

The present paper is a report about some experiments on dogs where simultaneous analyses of P_{CO_2} and pH of lymph and blood were made in the steady state. Thoracic duct lymph, collected anaerobically from a plastic tubing in the neck, has been compared with arterial blood with respect to P_{CO_2} and pH and the ventilation has been varied by intermittent positive pressure ventilation and an artificial "metabolic" acidosis has been provoked by intravenous hydrochloric acid.

Techniques

Sampling

All dogs weighing 10—15 kg were anaesthetized with intravenous nembutal. A fairly deep level of anaesthesia was maintained to facilitate the surgical procedure. In a first series of six dogs the thoracic duct was ligated close to the venous entrance and the main branch was cannulated. Special care was taken to avoid any lesion to the pleural cupola. In three dogs the thoracic duct was cannulated inside the thoracic cage according to the technique described by CARLSTEN, KAHLSON and WICKSELL (1949). By means of a short plastic tubing the lymph was collected anaerobically over redistilled mercury and at intervals transferred over into a glass syringe. The dead space in the syringe was filled with saline-heparin solution and a droplet of mercury.

Blood was collected in air-tight heparinized glass syringes from the femoral artery using an indwelling plastic catheter. The intra-arterial blood pressure was measured with a strain-gauge manometer.

Methods

All animals were tracheotomized and the tracheal tube was inserted into an incisure in the midportion of the thrachea.

Intermittent positive pressure breathing (IPPB) was performed by a respirator (type AGA). Peak inspiratory pressure of 180 mm H_2O was always used and the inspiratory phase is about one third of the total cycle. The respiratory rate was 14—16 a minute at ventilations of about 4 l and 32 at minute ventilation of 8 l.

In some dogs the oxygen content of the inspired gas was varied from 13 to 50 per cent O_2 in N_2.

In those dogs where the thorax was never opened expired gases were collected at intervals in Douglas bags and analyzed for oxygen and carbon dioxide content. P_{CO_2} and pH of lymph and blood were measured within 1 hour after sampling with a device invented by STÄLLBERG-STENHAGEN and similar to that described by JÖRGENSEN and ASTRUP (1957). The samples, in small tonometers, were exposed at 37° C to gases saturated with water vapour and a known CO_2 tension. From these the samples were drawn by suction through capillaries into the measuring chamber, where pH was measured at 37° C (Radiometer pH M3). pH and P_{CO_2} were plotted on a semilog paper and the actual P_{CO_2} was read from the calibration line at the pH measured in the sample before gasequilibration. The pH measurements by this technique contain an error of a single determination on lymph or blood of 0.0055 ± 0.0005 pH units. This value was calculated from 51 duplicate determinations on a number of the dogs. P_{O_2} of arterial blood was measured polarographically according to BARTHELS (1951) with a dropping mercury electrode.

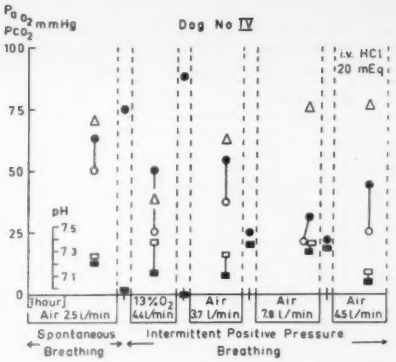

Fig. 1. △ P_{O_2} o P_{CO_2} □ pH. Open symbols arterial blood, filled symbols thoracic duct lymph. Dog no. IV. The P_{CO_2} and pH difference between arterial blood and thoracic duct lymph is a consistent finding irrespective of ventilation and arterial oxygen tension. The quick response to further impairment of ventilation on lymph pH and P_{CO_2} is demonstrated by samples taken when changing to artificial respiration and other gas mixtures.

Procedure

After the completion of the surgery the dogs in the first series were allowed to breath spontaneously and when a steady state was established expired gases were collected in Douglas bags and at the same time blood samples were taken. Because of the large amount of lymph necessary for the PCO_2 determination (15—20 ml) the period of lymph sampling was longer than that for blood and air. The dogs were then switched to artificial respiration using a respirator.

In the second series samples were taken only during positive pressure breathing. A steady state under the different experimental conditions was supposed to be established after half an hour. Lymph flowing in periods, before steady state was reached, was as a rule discarded but some such samples were analyzed and are given as transitional values between two different conditions (Fig. 1). Different gas mixtures were used and the ventilation was varied in order to study the effect of respiratory acidosis and alkalosis. Loss of fluid was replaced by subcutaneous injection of saline.

In two dogs "metabolic" acidosis was induced by injecting 2 ml 1 N HCl in 40 ml saline.

Results

Table I gives all relevant data. Dog I is excluded from further consideration because in this case lymph was analyzed one day after blood.

The present comparison between lymph and arterial blood is only made in the steady state. The difference between pH in blood and lymph, which is illustrated in Fig. 1, dog IV, is 0.12 pH units and is statistically significant ($p<0.001$). Fig. 1 gives a schematic survey of a typical experiment. The first data are from spontaneous breathing, when the dog is obviously hypoventilated presumably due to the anaesthesia and the surgery. The transitional value of lymph CO_2 tension was obtained during the preparation when the hypoventilation was further exaggerated and showed an increase from 63 to 75 mm Hg, indicating the rapid response in lymph P_{CO_2} to impaired ventilation.

Table I.

Dog	Gas mixt. Breath. pattern	Vent. l/min.	Resp. freq.	$P_{a}O_{2}$ △	$P_{a}CO_{2}$ ○	$P_{I}CO_{2}$ ●	pH_{a} □	pH_{I} ■
I	Air							
	spont. breath	—	—	51	33	[1]102	7.35	[1]6.91
	18 % O_2							
	IPPB	3.9	—	49	52	[1]120	7.32	[1]6.83
II	Air							
	spont. breath	—	8—10	70	49	78	7.26	7.13
	Air							
	spont. breath	3.5	8—10	—	40	60	7.33	7.19
	13 % O_2							
	IPPB	7.3	15	24	17	35	7.45	7.37
	50 % O_2							
	IPPB	7.8	23	132	22	32	7.45	7.36
III	Air							
	spont. breath	1.7	12	(74)	64	76	7.17	7.14
	Air							
	IPPB	3.8	16	80	26	60	7.18	7.07
IV	Air							
	spont. breath	2.5	8	71	50	63	7.25	7.21
	13 % O_2							
	IPPB	4.4	14	36	25	50	7.37	7.12
	Air							
	IPPB	3.7	16	63	37	54	7.27	7.10
	Air							
	IPPB	7.8	32	76	21	31	7.36	7.29
	Air							
	IPPB	4.5	16	76	25	44	7.13	7.05
V	Air							
	spont. breath	2.9	18	50	47	84	7.26	7.03
	Air							
	IPPB	4.6	18	66	40	72	7.25	7.13
	13 % O_2							
	IPPB	6.1	26	34	24	46	7.30	7.25
	Air							
	IPPB	7.4	32	60	42	37	7.33	7.23
	Air							
	IPPB	6.2	30	100	26	45	7.05	7.03
VI	Air							
	IPPB	—	15	59	45	50	7.30	7.29
	Air							
	IPPB	—	19	57	31	43	7.31	7.30

Dog	Gas mixt. Breath. pattern	Vent. l/min.	Resp. freq.	$P_{a_{O_2}}$ △	$P_{a_{CO_2}}$ ○	$P_{l_{CO_2}}$ ●	pH_a □	pH_l ■
VII	Air spont. breath	2.0	6	—	56	82	7.24	7.09
	Air IPPB	—	16	—	22	46	7.48	7.31
	Air IPPB	—	16	—	24	51	7.49	7.24
IX	Air IPPB	—	10	—	18	43	7.44	7.23
	Air IPPB	—	30	—	17	31	7.40	7.34
X	Air IPPB	—	16	—	10	23	7.61	7.48

a = arterial
l = lymph
IPPB = intermittent positive pressure breathing
' = analyses of lymph performed after refrigeration for 24 hours

During IPPB with 13 per cent O_2 the minute ventilation was increased and this state is reflected in the decrease in $P_{a_{O_2}}$ $P_{a_{CO_2}}$ and $P_{l_{CO_2}}$. IPPB in air and at different minute ventilations also give predictable changes in P_{CO_2} and pH of arterial blood and the corresponding data of lymph are changed in the same direction but with a distinct systematic difference. The injected hydrochloric acid provokes low pH in both lymph and blood.

The observed constant pH difference between lymph and blood applies to variations in "acid-base" balance from respiratory origin as well as from experimentally induced "metabolic" acidosis.

Discussion

In the quiescent animal, thoracic duct lymph is derived mainly from the thoracic and abdominal cavities. The present lymph samples are thus not representative for the whole animal but contain mostly lymph from the intestinal tract and the liver.

Lymph in this study without any exception has a lower pH and a higher P_{CO_2} than arterial blood, confirming the results of GESELL (1929), but in contradiction to KRÜGER (1925) and HEIM and LEIGH (1935). The last mentioned authors analyzed cervical lymph, *i. e.* extracellular fluid mainly from

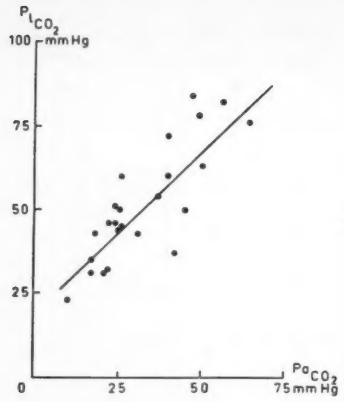

Fig. 2. The relationship between P_{CO_2} in arterial blood and thoracic duct lymph. Regression line represents the equation $P_{lCO_2} = P_{aCO_2} + 18.3$.

skin and muscles, and thus of a different composition to thoracic duct lymph and this may explain the difference, whereas KRÜGER's data also applied to thoracic duct lymph.

It may be concluded from the present experiments that the relationship between P_{CO_2} in blood and lymph stays fairly constant irrespective of ventilation, oxygenation and induced acidosis. Under the different experimental conditions both blood and lymph show concordant variations. This conclusion is valid for all the experiments and the relationship between P_{CO_2} in arterial blood and lymph is shown in Fig. 2. From the regression line calculated ($Y = 1.0\,X + 18.3$) the P_{CO_2} difference between arterial blood and lymph is approximately 18 mm Hg.

Blood perfusing the intestinal tract has a low arterio-venous oxygen and carbon dioxide difference. It seems reasonable that a large A—V difference in respect to O_2 and CO_2 should accentuate the present observed difference in P_{CO_2} between lymph and arterial blood. This fact makes the observed arterio-lymphatic P_{CO_2} difference more remarkable and is an inducement to undertake further tissue gas studies in comparing, within circumscribed areas, arterial, venous and lymphatic gas tensions. According to this hypothesis lymph from the lungs should have a low P_{CO_2}. This seems to be true as shown in a preliminary study on gas tensions in lymph from the right lymphatic duct (CARLSTEN and SÖDERHOLM, to be published).

The present experimental procedure with lymph sampling from the thoracic duct precludes part of the normal mixture of blood and lymph. It is reasonable to predict that arterial blood would have had a somewhat lower pH if normal lymph flow had been intact. This error cannot be calculated in detail but must presumably be insignificant due to the relatively low lymph flow per unit time.

Arterial blood P_{CO_2} and pH reflect the gas tensions in the different body compartments only qualitatively. The observed difference in P_{CO_2} and pH between arterial blood and lymph is of a higher order than was expected from the known arterio-venous difference and indicates that the gas tensions on tissue levels deviate definitely from those presumed from data of arterial and venous blood, as well as from those calculated from subcutaneous gas pockets.

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From the Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, Sweden

Some Types of Response of Single Cells in the Rabbit Lateral Geniculate Body to Stimulation of the Retina by Light and to Electrical Stimulation of the Optic Nerve

By

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Abstract

ARDEN, G. and Y.-M. LIU. *Some types of response of single cells in the rabbit lateral geniculate body to stimulation of the retina by light and to electrical stimulation of the optic nerve.* Acta physiol. scand. 1960. *48*. 36—48. — Recordings have been made from single cells in the rabbit's lateral geniculate body, using micropipette electrodes. Two extreme types of response to light can be distinguished. One has a low threshold and is more or less prolonged. The other has a higher threshold, and the discharge is shortened to one or two spikes followed, efter a pause by a delayed burst. Cells which respond with a short burst of spikes cannot follow repetitive stimuli, owing to inhibition. Most cells show evidences of inhibition, but the discharge characteristics can be altered by altering the stimulus. Electrical stimulation of the optic nerve at the chiasma results in post-synaptic firing with latencies which range from 1.6—3.5 msec, or longer.

The lateral geniculate body has been the subject of many studies of central nervous synaptic action (*e. g.* P. O. BISHOP 1953 and others). These studies were confined to the electrical stimulation of the optic nerve and retinal activity prevented by destruction of the eye. The impression gained from such recent studies in the cat is that single lateral geniculate cells act as simple mono-synaptic relays. However, work with the retina intact (COHN 1956 *a*, *b*, DE

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VALOIS, SMITH, KITAI and KAROLY 1958) has shown that more complex phenomena can co-exist and more complex phenomena have also been reported on electrical stimulation (BISHOP, JEREMY and MCLEOD 1953, EVARTS and HUGHES 1957 *a*, *b*).

When the retina is intact, the geniculate constantly receives a barrage of impulses — the resting retinal activity. The synaptic conditions might therefore differ from those disclosed by the investigations referred to above when the retina was destroyed. In addition, the impulses which arrive at the lateral geniculate after the adequate stimulation of the eye by light, can only be very inadequately imitated by stimulation of the optic nerve. In the former case the impulses are not synchronous and have a particular organisation. Though one might expect, on first premises, impulses to pass through the lateral geniculate relatively unmodified, this seems rather a waste of so large, and (in many mammals) so elegantly striated an organ. Indeed, the histology of the lateral geniculate allows for a great deal of cellular interaction, and several histological theories of lateral geniculate function have been advanced (for ref. see WALLS 1953).

Direct investigations of the visual functioning of the lateral geniculate have been few. BOHM and GERNANDT's (1950) pioneer investigation was not followed up. More recently COHN (1956 *a*) and ERULKAR and FILLENZ (1958) have reported post-synaptic interaction between the two eyes in cats, while DE VALOIS *et al.* (1958) have reported that the response to light in single cells of the monkey's lateral geniculate is variable and complex. We have tried to record responses to light in the rabbit's lateral geniculate and correlate these findings with electrical stimulation of the optic nerve. The rabbit's visual system has the advantage of simplicity. The retina is almost 'pure rod', the chiasma almost all crossed, and the lateral geniculate unstriated and fairly uniform. Our results agree with those of DE VALOIS, that lateral geniculate activity is both variable, confusing, and surprising, to a degree. Though the present results are not exhaustive we feel that the analysis, so far as we can take it, provides some substantiation and amplification of our knowledge of the lateral geniculate body of the rabbit.

Material and Methods

Pigmented rabbits weighing 2.5 to 3.5 kg have been used, since albino rabbits seem occasionally to possess defective ERG's (ELENIUS 1958). However, small areas of retinal degeneration seem quite common in all rabbits (TANSLEY, personal communication).

The animal was anaesthetized with ether, and spinalised at the level of C1 to C2: the blood pressure being supported by one injection of isopropylnoradrenaline (isodrine, 2 per cent, 0.5 to 0.6 ml intramuscularly) just before the cord was cut. Occasionally another injection was given toward the end of the day. Atropine was often given as a premedication (0.5 ml 1 per cent intravenously). Mydriasis was achieved by the instillation of atropine and isodrine. All wounds and pressure points were locally anaesthetized. Other drugs were given through an intravenous cannula. After spinalisation

the animal was allowed to recover, mounted in a head holder, and two small holes bored through the cranial vault topographically over the chiasma (H-C plane A3) and the lateral geniculate body (H-C plane P5, L7) (SAWYER, EVERETT and GREEN 1954). The dura was incised and removed. The eyelids were held by stay sutures. A plastic contact lens was used to protect the cornea. Additional immobilisation was secured by Flaxedil (4 — 5 mg/kg/90 min, intravenously), or by urethane (1 g/kg, 1/2 intraperitoneally and 1/2 intravenously). Neither of these drugs affected the activity of the geniculate cells significantly. They served to prevent the eye and other movements that the *encéphale isolé* shows.

We preferred to use the *encéphale isolé* since the preparation was more stable. In particular, pulsations of the brain due to respiration and the arterial pulse were diminished after cisternal drainage.

The animal was allowed to dark adapt for at least 90 min before the experiments began.

Electrodes. Stimulating electrodes were two fine entomological steel pins, insulated to their tips, which were sharpened to a diameter of a few μ. These were introduced through the hole above the chiasma and lowered till they reached the chiasmal level. Such small electrodes did not appear to interfere with the geniculate responses to light stimuli, but when, in early attempts, we tried to place electrodes round the optic nerve in the orbit the eye was always damaged.

The recording electrodes were glass micropipettes filled with 4 M NaCl. The tip resistance varied between 5 and 20 MΩ. The higher resistance electrodes were noisy and tended to break more easily. The recording electrode was thrust through the brain via the hole over the lateral geniculate until the correct place was reached. We found the geniculate to be a relatively stable part of the brain and were often able to hold a spike for an hour or two. However, the system did not compare with the rigidly closed techniques (MOUNTCASTLE, DAVIES and BERMAN 1957 and others). Our spikes were mostly of small size, 200 to 2,000 μV: attempts at further approximation of the micropipette tip invariably led (if the spike grew larger) to injury discharge. The larger the spike, the shorter the time for which we could hold it.

Histological check. The position of the microeletrode tip was roughly determined by withdrawing the micropipette and noting the depth of penetration. An entomological pin insulated to the tip was then inserted to, as nearly as possible, the same point. Iron was deposited anodally and the position identified by the ferrocyanide-formalin method. The accuracy of this method is not great but was good enough for our purpose.

Recording and display. Potentials were recorded via cathode follower and RC amplifier in the usual way. A photocell monitored the light-flashes. Its output could be displayed on the cathode ray oscilloscope and also triggered the time base. Two tube faces were available: on one we displayed spikes, while the other synchronously displayed the photocell output. In many experiments the spikes were made to trigger a pulse applied to the Z axis of the cathode ray oscilloscope, each spike appearing as a bright dot on the sweep which displayed photocell output. This technique enabled the results of many stimuli to be easily compared and evaluated. It had the disadvantage that only the bigger spikes could be used. Furthermore, it could not be employed when two spikes were recorded simultaneously. The same system was used as a crude sort of beam-brightener. The amplifier output was monitored by a loudspeaker.

Light system. The light source was a 24 V, 15 A tungsten ribbon lamp run at a constant colour temperature of 2,760° K. A system of lenses passed the beam through steel powder filters (Bausch and Lomb) and a neutral wedge (Ilford), the densities of which have been repeatedly checked by successive workers in this laboratory. Our own calibrations showed insignificant changes from rated values. The final image of the filament

was focused on the rabbit's cornea so that it 'saw' in Maxwellian view a bright image of the last lens, which subtended about 15° solid angle. The last filament image was smaller than the dilated pupil. We tried to illuminate a central portion of the retina below the white streak of myelinated nerve fibres (see THOMSON 1953). The rabbit's hypermetropia and irregular errors of refraction remained uncorrected, so that, with these and the imperfect media, the image on the retina must have been considerably larger than that calculated on a geometric basis.

Energy calibration. The intensity of the light was calibrated by direct comparison photometry at different intensity levels. These gave an illumination of 180,000 lux. Such a calibration neglects the different apertures of the rabbit and human eye.

Results

As the microelectrode was thrust through the brain, the first nuclear mass reached was the hippocampus, which was easily recognised by the discharges which we encountered in quick succession, due to the closely packed cells. Some of these were light sensitive, and responded to flicker. The microelectrode then passed through rather an 'empty' layer, until at about 7 mm beneath the surface, the lateral geniculate was reached. The first activity encountered was the nerve spikes of the optic tract, firing with the 'on' or 'off' (or both) of the flash. They were always very small, and we could not isolate them.

In the substance of the lateral geniculate, we were not always able at first to find an area in which the cells responded constantly to light. A histological check showed that the electrode was in fact in the lateral geniculate. Sometimes we had to hunt for a long time before finding an active part of the lateral geniculate. In such an active part, many more cells could be recorded.

The usual finding was of one large spike, commonly initially positive, which was recorded together with small spikes, initially negative. Though the smaller spikes were usually only a few hundred μV in size, several elements could be distinguished by their difference in spike size.

A large spike could often be isolated with great ease and then it was common to find that, like the cells in the inactive part of the lateral geniculate, it responded poorly to light. The resting discharge was usually irregular and consisted of high frequency bursts of 2 to 5 spikes, separated by irregular pauses. The response to light was fitful and had a high threshold. In contrast, the little spikes had a lower threshold and a much more constant response to light. When one of them was (with some difficulty) isolated it was found that though such a cell did not respond in a completely regular fashion, in general there was less variation of the discharge than was the case for the big spikes. Some cells of this type, however, responded absolutely invariantly to a constant stimulus. We gained the impression that there were clusters of the cells associated with the small spikes and the cells associated with the big spikes were scattered amongst them (see TABOADA 1927—28).

Fig. 1 shows a record in which these two sorts of spike were recorded simultaneously. The stimulus is a slow flicker. The cells are off-elements. Four

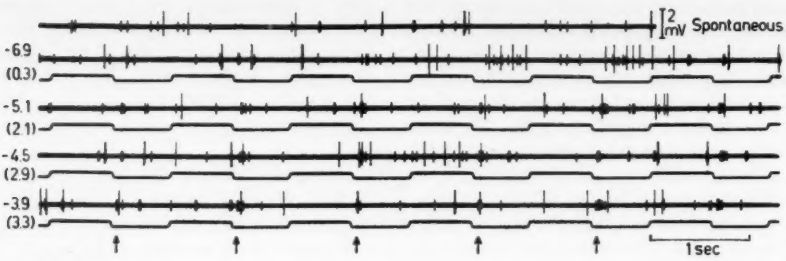

Fig. 1. Response of two spikes, simultaneously recorded, to slow repetitive flashes of different intensity, expressed as, in the brackets, log units above threshold of the more sensitive small spike. Absolute intensity given as log units below full light intensity. Upward deflection of the photocell trace signals 'on'; upward deflection of spike is positive. Same conventions adopted in all subsequent figures. Note the small spike responds regularly to every 'off', the big spike responds only irregularly.

intensities of light were used. The lowest was about 0.3 log units above the threshold of the small spike. At this intensity the small spike responded to every 'off' with a short train of impulses and did so at all light intensities, the latency decreasing with intensity. The big spike, on the other hand, was evoked by only three or four of the six flashes, usually with a single discharge; and it continued to fire in this haphazard way up to a light intensity 3.3 log units above the threshold of the small spike. It is impossible to define a threshold for such a cell. In sum it may be said that this figure gives an accurate idea of the responses of the lateral geniculate cells to stimuli widely separated in time.

High threshold irregular discharges. While the high threshold and inconsistency of response might be expected in cells located in a part of the lateral geniculate not connected directly with the illuminated retina, such discharges are also commonly found in the most active parts of the lateral geniculate. Since according to BROUWER (1927), there is a fairly good topographical projection of the retina on the lateral geniculate it is most unlikely that neighbouring cells (as shown in Fig. 1) should receive optic nerve afferents from widely separated parts of the retina. It might be thought however, that the difference in response was due to the fact that the cells received afferents from that part of the retina on which was located the edge of the bright image of the last lens. The difference in response might therefore be due to the difference in the illumination of neighbouring areas of the retina. The extended threshold shown by the big spike of Fig. 1 is not however, what one would expect if it was fed by a part of the retina receiving mainly scattered light, since the latter will increase in proportion to the intensity of the main light beam. The hypothesis can be disproved by placing a piece of opal glass in front of the eye. This forms a new and much larger image which is now projected on the retina. If this is done at a moderate light intensity, say 3 log units above the usual threshold of the small spike no change occurs in the discharge of these 'irregular' cells.

It gradually became our practice to select for study only those regions of the lateral geniculate in which both sorts of response could be recorded.

The big spikes might also be thought to arise from cells whose dendritic field was

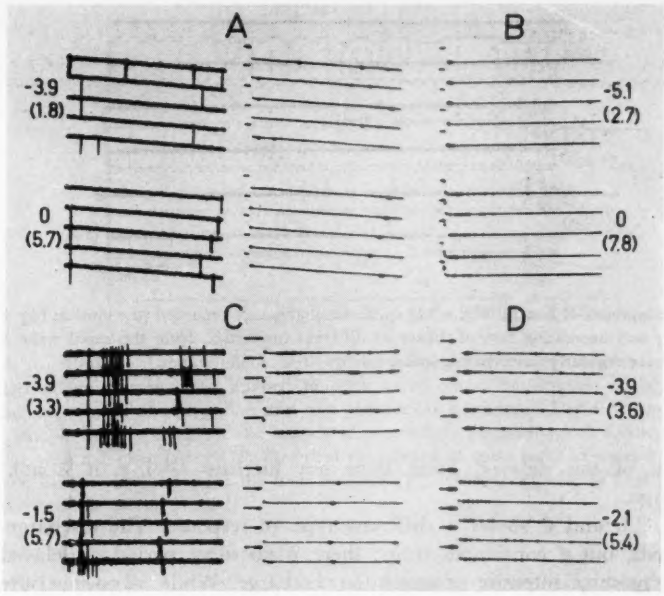

Fig. 2. Discharge pattern of different geniculate cells to single flashes of different intensity. For cell *A* and *C*, parallel records of spike and photocell-dot traces shown (see Methods). Trace triggered by flash; duration: *A* and *B*, 20—22 msec; *C* and *D*, 100 msec. *A*. Initial response consists of a single spike, followed by cessation of all spontaneous activity and then a rebound discharge. *C*. At low intensity (3.3), discharge pronounced and less inhibition than *A*. Discharge greatly inhibited at high intensity (5.7) and rebound more pronounced. *B* and *D*. Discharge prolonged at high intensity, no inhibition and no rebound.

partially destroyed by the close approximation of the microelectrode (this would also account for the size of the spike). This supposition is unlikely to be true since we could often hear, from a distance, the irregular discharge of such a cell and the regular flickering discharge of the other types of unit, while the microelectrode was advanced to a position where the spike size grew large enough to permit recording: and the two sorts of response persisted unchanged. If a cell with a regular discharge was isolated, its response remained constant till, on further advance of the microelectrode, injury discharge began.

The response of lateral geniculate cells to single flashes of light is usually very brief; many cells respond with one or two spikes. Following this, the cell is often silent for a period of about $^1/_2$ sec., when it fires again. Such discharges are shown in Fig. 2 a and c. Notice that an increase of intensity either does not alter or greatly diminishes the number of spikes discharged initially but the delayed burst is augmented. This later discharge has a very variable latency, even at the higher intensity. If a longer flash is used, the

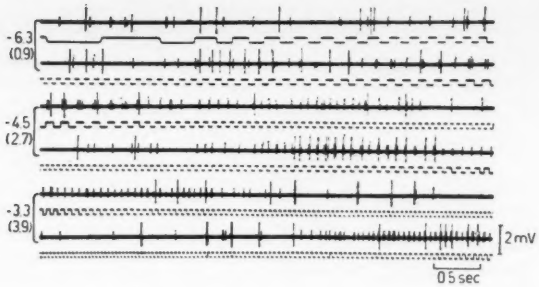

Fig. 3. Responses of a small and a big spike simultaneously recorded (the same as Fig. 1) to increasing and decreasing rate of flicker at different intensities. Note the small spike responds much more regularly than the big spike.

latency of the delayed burst does not increase — i. e. it is not an off discharge.

Fig. 2 b and d shows a different type of response. The discharge is not in bursts, but a continuous train; there is no silent period or delayed burst. An increase of intensity prolongs the discharge. While all variety of response patterns can be observed in the cells of the lateral geniculate, it is possible to make a subdivision on the basis of the characteristics shown in Fig. 2. The more sensitive cell responded with a train of impulses. The cell which responded poorly to light had a short discharge, pause and delayed burst. The shorter the evoked discharge, the more irregular the response to light.

Flicker and fusion frequency. Cells with brief irregular discharges cannot follow repetitive stimuli. This is shown in Fig. 3, where the same two spikes as in Fig. 1 are shown, evoked by flicker of various frequencies and intensities. Note that the big spike scarcely follows the flicker at all. The flicker fusion frequency is lower than that for the small spike even though it is impossible to determine accurately. The flicker-fusion rates of such poorly responding cells did not seem to alter significantly with intensity.

The response to electrical stimulation of the optic nerve. Even with microelectrodes of the tip resistance used here, it was usually possible to record with high amplification, the small summed potentials ascribable to the synchronous pre-synaptic activity evoked by an electrical shock. Following this the post-synaptic responses of the cells near the electrode were recorded. Fig 4 *B* shows such a record. The stimulus, which is above threshold, produces a large artefact, which merges with the deflection due to the tract response (T). Following this there is a negative deflection, which is the summed response of a few small negative spikes (S). At I one big spike is recorded. We found that even when a small spike had been isolated, with light as a stimulus, an electrical stimulus ob-

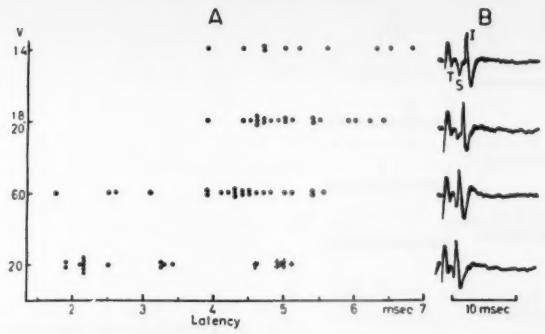

Fig. 4. *A.* Abscissa: Latency. Each dot represents one separate determination. Ordinate: Output voltage of stimulator, applied to optic nerve. Note the decrease of latency with increase of strength of stimulation and two preferential latencies of 2 and 3.3 msec for the 20 V stimuli. Crosses represent the latency of a repetitive response which follows the early response with a 2 msec latency. *B.* Electrical stimulation of optic nerve at suprathreshold intensities. Compound potential is the tract response (T), summed response of 2—3 S cells (S) and the response of one I cell (I).

scured the record by activating, synchronously, many neighbouring cells. The effects of electrical stimulation have therefore only been investigated in the larger spikes (I of records 4 *B*) which usually belonged, as described above, to cells which responded poorly to light. The electrical latency of the small grouped spikes (S, 4 *B*) varies between 1.8 and 2.2 msec, and is followed by an abrupt deflection as the big spike develops. Its latency is variable and ranges in the figure from 2.0 to 3.4 msec. This is near the lower limit of latencies recorded after stimulation of the optic nerve. The latency varies both for different shocks and with stimulus strength, as shown in Fig. 4 *A*. These results are from another experiment. At a 50 per cent response intensity of stimulation (1.4 V), the latency varies in different trials from 3.8 to 6.8 msec and decreases with increase of stimulus intensity. The strongest stimulus evokes spikes with latencies of either approximately 2.0 or 3.3 msec and there is also a repetitive discharge (crosses) of a latency of approximately 5 msec. Lower stimulus strengths evoked spikes with a much longer and more variable latency. The longest latency we encountered was 25 msec. The two shortest latencies, 2.2 and 3.3 msec occurred with great regularity, if suprathreshold stimuli were employed.

The conduction rate in the optic nerve of the rabbit was measured by Granit and Marg (1958). The two main fibre groups were shown to have conduction velocities of 55 to 60 m/sec, and 23 m/sec respectively. Since the stimulating-recording distance in our experiments was only 21 to 23 mm, differences in conduction rate cannot account for the two preferential latencies.

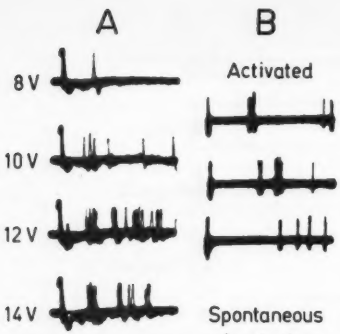

Fig. 5. The response of relatively light-insensitive cell to stimulation of optic nerve. Eight superimposed traces for each row. *A*. Shows 3 3 msec response and repetitive discharge. *B*. Compressed time scale. Note the prolonged pause and the variable latency rebounds. The stimulus artefact and the response appear as a thick line on the left. Stimulus 20 V. 'Spontaneous': Similar superimposed traces without stimulation serve as control.

Fig 5 *A* shows 8 superimposed traces in each case. For the weakest stimulus, 8 volts, the cell responded only in one trial and the absence of spikes shows the spontaneous activity to be low. All the spikes in the other sweeps are evoked by the stimulus. The latency of the first spike after the artefact decreases slightly as the stimulus strength is increased, but does not fall below a value of 3.3 msec at 18 V. Strikingly, the variation in latency in successive trials decreases with increase of stimulus strength, and at higher intensites, the delayed response decreases.

Fig. 6 *A* and *B* shows a 2 msec latency. Each sweep of *A* and *B* consists of 10 superimposed traces. *A* and *B* were taken within a short time of each other, and the record shows how variable were the responses of the lateral geniculate, even to electrical stimulation. At the two lowest strengths of stimulus, the cell responds with a long and variable latency. As the stimulus strength is increased, 2 msec latency spikes are discharged, and the delayed group increases in number.

In our arrangement, tiny stimulating electrodes were placed somewhere close to the chiasma, in order to avoid damage to the optic nerve fibres, and the threshold intensity of stimulation was of the same order as the maximum output of the stimulator. We are therefore unable to say whether or not all long latency spikes could be driven down to less than 2 msec, if sufficiently strong stimuli were employed.

We gained the impression that irregularity of light evoked discharge was associated with a long latency to electrical stimulation.

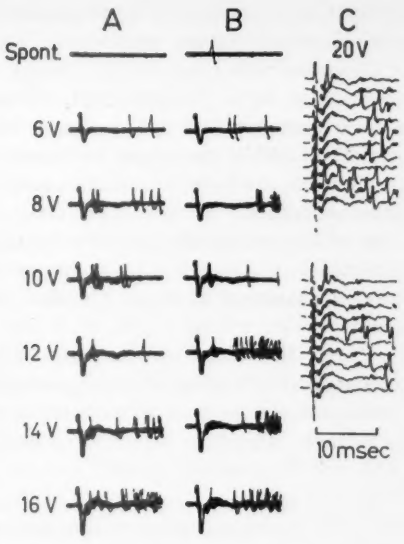

Fig. 6. Optic nerve stimulation. *A* and *B*. Superimposed traces (10—11). Note a first order response, variation of response with stimulus strength and with identical stimuli. *A* and *B* were obtained successively. *C*. Two examples of the superimposed records displayed on a continuous paper, showing the refractoriness of the first order response to successive stimuli at a frequency of about 10/sec.

Inhibition. Sometimes we were unable to drive a spike from the optic nerve, though it was sensitive to light. Because our electrodes were so fine and placed blindly, we cannot say whether this was because of a failure to stimulate the pre-synaptic fibres proper to the cell, or if simultaneous inhibition prevented the discharge. Single shocks to the optic nerve certainly produced inhibition, for following the initial discharge of spikes, a prolonged silent period occurred as the superimposed traces of Fig. 5 *B* show. This sort of inhibition may also explain the fact that the cells respond in a variable fashion to successive stimuli. Fig. 6 *C* shows the successive responses of the same cell as in Fig. 6 *A* and *B*. The optic nerve was tetanised at about 10/sec. The cell responds to only a few of the shocks with a short latency. However, if the rate of stimulation was reduced to 3/sec (not illustrated) the short latency spike occurred every time. The later discharge can occur with a tetanus, even when the short latency spike is not evoked.

Discussion

Two types of response. Though some lateral geniculate cells can respond with a long train of spikes, the usual evoked response is brief, followed by a long silent period, and terminated by a delayed burst.

This sequence suggests that, following the exitation transmitted from the retina, the cell is inhibited. The fact that such cells are relatively insensitive

to light, only respond to a proportion of stimuli and cannot follow repetitive stimuli, re-inforces this suggestion.

Correlation with retinal activity. Though cells exist in the retina which are not sensitive to light (GRANIT 1941, KUFFLER 1953, LENNOX 1959) published accounts suggest that they are more scarce than similar cells in the lateral geniculate. While the reason for the presence in the retina of insensitive cells is unknown, the bulk of insensitive geniculate cells do respond if the net difference between inhibition and excitation is great enough, though the modification of the resting discharge by the light flash may be small. In general all geniculate responses tend to be phasic. We have seen no cell whose discharge was as prolonged as those THOMSON (1953) described, in the rabbit's optic nerve.

Relationship to other work on higher afferent pathways. DE VALOIS *et al.* (1958) in monkeys and TASAKI, POLLEY and ORREGO (1954) in cats have reported findings similar to those of this paper in their study of unit activity in the lateral geniculate. The long inhibitory pauses in particular are commented upon. The medial geniculate activity also corresponds in many ways to the present findings especially in the brevity of the responses evoked, and the inability of the cells to follow repetitive stimulation (VERNIER and GALAMBOS 1957). In the optic cortex, JUNG, CREUTZFELDT and BAUMGARTNER (1955) have reported extensive series of phenomena, which resemble very closely the activity of the lateral geniculate. Of particular interest is the 'A' response type described by JUNG. Such cells do not respond to a single flash but follow a flicker by a modification of the rhythm of the basic discharge. They number about 40 per cent of all cells and are ascribed a 'fonction stabilatrice'.

Two types of cell? The responses of Fig 2, in their extreme forms seem preferentially associated with the two types of extra-cellular potential. This is in harmony with the findings of MOUNTCASTLE *et al.* (1957) in the somato-sensory cortex. There, a smaller proportion of positive extra-cellular spikes were associated with 'easy driveability' (from afferent stimuli) than were the smaller negative spikes. MOUNTCASTLE *et al.* (1957) believed this to be due to damage of the cells which gave the positive extra-cellular spikes. We have some evidence that this will not explain our results. Indeed, VON KOELLIKER (1896) described the presence in Golgi preparations of rabbit lateral geniculate, of two types of cells. The first, the 'Strahlenzell', was large, solitary, and possessed a recurrent collateral. The second, the 'Buschzell', was smaller, of a different form and occurred in clusters. Though it is tempting to identify our two types of spikes with cell types, they may equally well be due to some other characteristics of the site from which we record.

Electrical stimulation. The outstanding finding on stimulating the optic nerve is the long latency of some of the evoked spikes. This is unsuspected if massed responses are recorded. However, P. O. BISHOP (personal communication) has made similar observations.

The production of inhibition by optic nerve stimuli resembles the effect of light, and may be powerful enough to prevent repetitive supramaximal shocks from being followed.

The 3 msec latencies which can occur so regularly might be explained by supposing that there may be an additional synapse between the lateral geniculate cell which is investigated, and the optic tract terminals. This is surprising since all anatomical evidence from retrograde and transsynaptic degeneration studies (GLEES 1941) indicates that there are no true interneurones in the lateral geniculate. In the cat, there is physiological evidence for the existence of closed chain neuronal linkages (P. O. BISHOP *et al.* 1953). If similar mechanisms exist in the rabbit these easily explain the repetitive responses to an electrical shock, and if the chains are not cell specific, their presence would explain the longer latencies we have observed.

The fact that the response to light often closely resembles the response to an electrical shock (brief burst, pause, delayed response) implies that the form of the geniculate discharge is not entirely determined by the incoming optic nerve action potentials.

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Some Responses of the Lateral Geniculate Body of the Rabbit to Flickering Light Stimuli

By

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Abstract

ARDEN, G. and Y.-M. LIU. *Some responses of the lateral geniculate body of the rabbit to flickering light stimuli.* Acta physiol. scand. 1960. *48.* 49—62. — The response of geniculate cells to flickering light shows differences from the responses of retinal ganglion cells. The evoked discharge is usually only loosely synchronised with the flicker and consists of a burst of impulses similar to a resting burst. In most cells both excitatory and inhibitory processes determine this discharge. Cells may follow a flicker by rhythmic inhibition of their resting activity. Alteration of the intensity of the stimulus does not greatly affect the number or frequency of the evoked spikes, but considerably alters the degree to which the spikes are synchronised with the flicker. These findings are discussed in relation to retinal electrophysiology and the way information is transmitted by the geniculate body.

The preceding paper (ARDEN and LIU 1960) described the response of cells in the rabbit's lateral geniculate body to single light flashes. It was concluded that the responses, which varied considerably from cell to cell, were determined by both inhibitory and excitatory processes. In the experiments of this paper this problem was studied using flickering light as a stimulus. This method

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was found to have certain advantages. The resting discharge of cells of the lateral geniculate is irregularly distributed. The response evoked by a single flash is often brief, and consists of a burst of spikes indistinguishable from the resting discharge. When the stimulus is a flicker, interference from the resting activity is reduced, and some types of quantitative treatment become more easy to apply. It was found that most cells responded to a flickering light stimulus by a synchronisation of their discharge with the stimulus. This synchronisation improved as the stimulus intensity increased. The number and frequency of the evoked spikes showed less alteration as the intensity increased.

In some respects, the present findings are different from those reported by authors who have studied retinal responses, though the latter must determine the responses of the geniculate cells to light. Retinal responses to flicker were studied by ENROTH (1952). The following summary of the salient points from Enroth's work may help to clarify the present results, and contrast the difference between geniculate and retinal flicker mechanisms.

A typical retinal ganglion cell responds to a light flash with a prolonged discharge. It responds to a train of flashes, presented as a flicker, by a series of short bursts of impulses, one burst per flash. The discharge to each flash is curtailed by the pre-excitatory inhibition of the succeeding flash. The first flash of a flicker may still evoke a prolonged discharge which is only later 'chopped' and follows the flickering light. The latency of the response to succeeding flashes is either constant, or varies gradually. The frequency of the initial discharge is correlated with fusion frequency.

Methods

Rabbits were lightly anaesthetized with urethane (1.5 g/kg/day). The rabbit's retina seems sensitive to falls in blood pressure (THOMSON 1953) and therefore it was felt that results on *encéphale isolé* animals, presented previously (ARDEN and LIU 1960) might be misleading. However, in control experiments with *encéphale isolé* similar responses were found, though unless the *encéphale isolé* shows much spontaneous movement, the resting activity is less than in the anaesthetized preparation.

Recording. Extracellular spikes of up to 2 mV were recorded with micropipettes filled with 4 *M* NaCl. The tip resistance varied from 5—30 M Ω. Spikes were amplified and displayed conventionally.

Stimulation. The ribbon of a constant voltage calibrated lamp was focused on the cornea. Intensity was altered with steel powder filters and wedges. Flicker was produced by a rotating sectored wheel (90°), which, placed at a focus of the light beam, produced a one-to-one light/dark ratio. The wheel was driven by a velodyne motor-generator.

Light intensities, unless otherwise stated, are in log units *below* the maximum available (about 180,000 lux), *i. e.* 0 = 180,000 lux, — 1, = 18,000, and so on). A fuller description will be found in the preceding paper (ARDEN and LIU 1960).

Results

Responses unaffected by inhibition. Cells seemingly unaffected by inhibition are rare. Fig. 1 shows the responses of such a cell, to flickering light of three different intensities. The response differs from the retinal response in several important

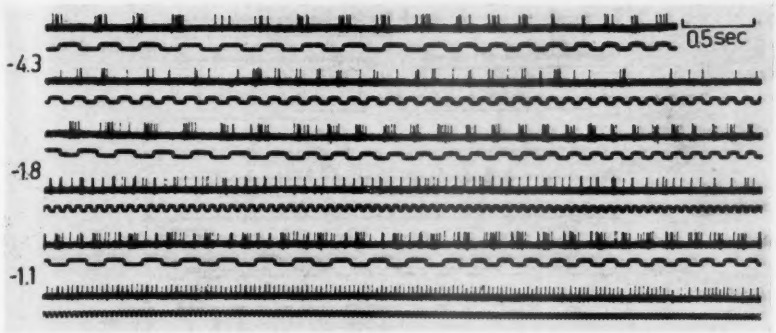

Fig. 1. Flicker responses of a cell unaffected by inhibition. Three light intensities are compared. Each row shows spikes and photocell record (up = 'on'). The upper of each pair shows a slow flicker, the lower, flicker near fusion frequency. At low flicker rates, the evoked responses contain high-frequency bursts, at all intensities. The discharge is prolonged by increase of intensity, and at —1.1 the slow flicker discharges merge. The fusion frequency rises with intensity. Spikes retouched.

ways. Even when a low intensity of light is used, sometimes the spikes are discharged at very high frequencies. The cell's resting discharge, and the evoked response, equally tend to occur in 'bursts'. When a low frequency flicker is employed, the duration of the discharge increases as light intensity increases. This process continues till at —1.1 log unit it is difficult to distinguish the responses to successive flashes, which are merged. (It must be emphasized that the responses shown are not the beginning of a flicker train.) This does not occur in the retina since 'pre-excitatory inhibition' separates successive discharges. Such a mechanism does not seem to operate in this cell. As the flicker rate increases, the evoked responses grow briefer, just as in the retina. Near fusion-frequency, the cell still produces a burst (most evident at intensity —4.3). This finding is unlike retinal discharges where the trailing and leading edges of successive excitatory and inhibitory processes clash, so that near fusion frequency only one spike/flash is evoked. The flicker fusion rate of this cell was high — approximately 25/sec at intensity —1.1 — and it increased as the intensity increased to about 40/sec (not shown in the figure). The flicker fusion frequency-intensity curve was very like the ERG flicker fusion curve given by ELENIUS (1958).

Responses dominated by inhibition. The type of response shown in Fig. 2, where there is no evidence of excitation at all, is also rare and is the antithesis of Fig. 1, though cells which show some evidences of inhibition are common.

The top row of the figure shows the activity in maintained light, and in darkness. In both conditions, the cell fires in rhythmic bursts and there is no difference between the two records. The cell (like almost all the cells investi-

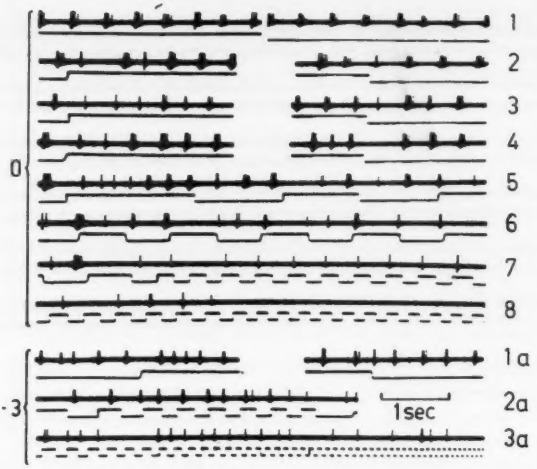

Fig. 2. Responses of a cell showing pure inhibition. Two light intensities are compared. *Intensity 0.* Row 1, rhythmic high frequency discharges unchanged in maintained light (L) and darkness (R). (Photocell records as in Fig. 1.) Rows 2, 3 and 4, the discharge falters following the beginning or ending of a long light flash. Rows 5, 6 and 7, response to flicker of increasing frequency. The regular resting discharge is broken up and the cell follows flicker. The average rate of firing decreases till in row 8, the cell is silenced. *Intensity —3.* Row, 1 *a*, single 'on' and 'off's produce little effect. Rows 2 *a* and 3 *a*, flicker decreases the resting discharge, but fusion frequency is higher, and the cell is not completely silenced. Note irregular latency of flicker responses.

gated) was affected by change of light intensity only. The succeeding three rows show the response to the beginning and ending of prolonged light flashes. There is a faltering of the resting discharge, but no spikes are evoked by the light. When a flicker of increasing frequency is used (rows 5—8) the resting discharge is progressively more and more inhibited, and the rhythmic inhibition modifies the discharge so that it 'follows' the flicker. The quicker the flicker the fewer the spikes discharged, till the cell is silenced: above fusion frequency the discharge returns.

When the light intensity is reduced, the inhibitory effect of change of illumination is reduced and the cell responds at higher flicker rates (rows 2 a and 3 a) and is never completely silenced. The optic nerve impulses which initiate the inhibition during a flicker, appear as a series of short trains of impulses. The greater effect of a more rapid flicker (compare row 6 with 7) must mean that the inhibitory effect of successive trains of impulses in the optic nerve summate, and produce a greater inhibitory action than could a single flash.

Mixed excitation and inhibition. There are many cells whose response is due to both excitatory and inhibitory processes. An example is shown in Fig. 3. At intensity 0, the cell does not respond to single flashes with a recognisable

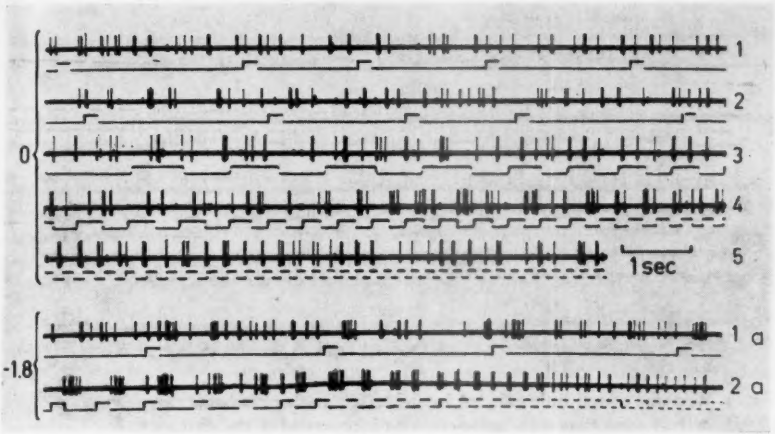

Fig. 3. Responses of a cell influenced by both excitation and inhibition. *Intensity 0.* Rows 1 and 2, single flashes do not evoke a recognisable response. Rows 3, 4 and 5, the cell responds in rhythm with the flickering light. Note irregularity of latencies. *Intensity —1.8.* Row 1 *a*, single flashes evoke a definite burst response. Row 2 *a*, the cell flickers with a regular latency to a higher fusion frequency than before. In row 2 *a* flicker is produced by a hand-operated shutter.

discharge. When the stimulus is a flicker, the discharge is modified so that it is loosely synchronised with the flickering light. It is very difficult to say what part of the flash produces the flicker (*e. g.* end of row 3, beginning of 4), and even at higher rates (rows 4 and 5) the 'latency' of the response is very inconstant. Note that the cell fires more often during the flicker, than in darkness. (Rows 1 and 2 may be taken as equivalent to the resting discharge.) This type of response indicates that though light facilitates the cell it is not 'driven'.

When the intensity is reduced, the single flash does evoke a discharge which is followed by a pause, and the cell follows the flicker with a more constant latency, and to a higher fusion frequency than before. The decrease in light intensity has revealed the excitation that was previously concealed, and the cell now follows the flickering light in a different, and more familiar way. The irregular synchronisation with the flicker at intensity 0 has no fixed latency. Were some part of the discharge an 'on' or 'off' effect, this should be apparent, since the latency of each successive discharge should be approximately the same as the one evoked by the flash prior to it; if, and only if, the correct assumption ('on' or 'off') had been made. Thus for example, if the group of spikes evoked by the flashes of row 5 are an 'on'-discharge, they should have a constant, or progressively and slowly altering latency, while if they are assumed to be an 'off' effect the calculated latency should change differently.

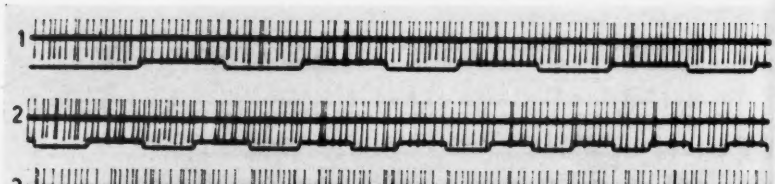

Fig. 4. Response of cell little affected by light. The resting discharge rate is very high. Approximately 50 msec after each 'on' there is a slight slowing of the discharge.

But the 'latency' measured in this way, shows very great variations, and though it becomes more regular when the flicker rate is higher this is equally true for all the alternatives, and the proportional change is still large. The shortest latency, approximately 100 msec is given by supposing that the bursts of row 5 are 'off' effects. But at this intensity cells which are driven in a more familiar way always have a much shorter latency; and in the other rows, the latency of this supposed 'off' effect drops below zero. This type of response is not peculiar to certain cells. A change in the intensity of the stimulus can change the type of response.

It is difficult to determine the flicker fusion rate of this cell (and of the cell in Fig. 3), since the discharge faltered at low frequencies; but it was about 6—12/sec in various cases. Notice that high frequency bursts are discharged near fusion frequency.

Fig. 4 shows the responses of a cell, which at first sight looks as though it were uninfluenced by the light. However, at about 50 msec after the beginning of almost every flash, a slight slowing of the discharge, followed by an even smaller acceleration, can be seen. Though this change of frequency is well within the range of spontaneous variation its more or less constant occurrence in a flicker train of increasing frequency, shows that it is connected with light, though the process eliciting the resting discharge is obviously more potent than the light-induced optic nerve input.

The discharge rate in this cell decreased only after the 'on' of the light. It is probable therefore that a pure retinal 'on'-discharge had an inhibitory effect on this geniculate cell. This phenomenon was often observed, and the behaviour of the inhibition could often be correlated with the characteristics of retinal

'on' flicker. For example, in some cells the discharge ceased at the beginning of the flicker. This time corresponds to that when the retinal 'on' cells produce a prolonged 'unchopped' discharge. In the succeeding flashes, the retinal cells should follow flicker. The geniculate cell response now appeared, but only as a single spike. As the rate of flickering increased (and the retinal 'on' discharges became briefer), the geniculate cell responses increased in duration, two or more spikes being discharged.

Alteration of discharge with intensity. An analysis was made of the changes in the response to flickering light as the intensity was varied. The investigation was confined to those cells, in which there was a definite response to each flash. The response was judged by listening to the loudspeaker. A slow flicker frequency was chosen (2.5—3.9 cps in different cases). If higher frequencies had been employed fusion-frequency would have prevented the analysis from being extended to low intensity, and, if lower frequencies had been used, the advantage of rhythmic stimulation would have been lost.

When a cell had been isolated, the light intensity was reduced till the response became indefinite. The light was then turned off, and the animal allowed to recover from any light adaptation. The resting activity and the responses to flickering light were recorded. Only if the spontaneous activity remained approximately constant throughout the experiment was the result accepted. The procedures did not alter the 'threshold' of the response by more than 0.6 log unit.

In frogs (HARTLINE 1938) the initial frequency of the retinal spike discharges increases with light intensity. This is true for pure visual purple elements in the cat (DONNER and WILLMER 1950). It only applies generally in the 'mixed' elements of the cat, since inhibition often increases with light intensity (GRANIT 1947, DONNER 1950). In the rabbit's predominantly pure rod eye (WALLS 1942) the initial frequency of discharge might be expected often to vary with intensity. An attempt made to investigate the initial discharge in geniculate cells proved, however, unfruitful, since the geniculate resting discharge (unlike the retinal — KUFFLER, FITZHUGH and BARLOW 1957) usually consists of sporadic bursts. The response to light also commonly consisted of similar high frequency bursts. The instantaneous frequency of discharge was meaningless (see Figs. 1, 3 and 6 and the histogram of Fig. 7).

THOMSON (1953) showed that the numbers of spikes discharged in rabbit optic nerve fibres following a light flash increased as the light intensity increased (investigated for responses within 2 log units above threshold). In the present flicker-experiments most of the spikes were discharged in response to light, and therefore, the number of spikes per flash could be estimated by measuring the average rate, taken over about 10 sec. This was only ambiguous at the lower intensities.

In many cases, when the stimulus is a slow flicker, the average rate of discharge is the same in darkness, and in flickering light (see ARDEN and

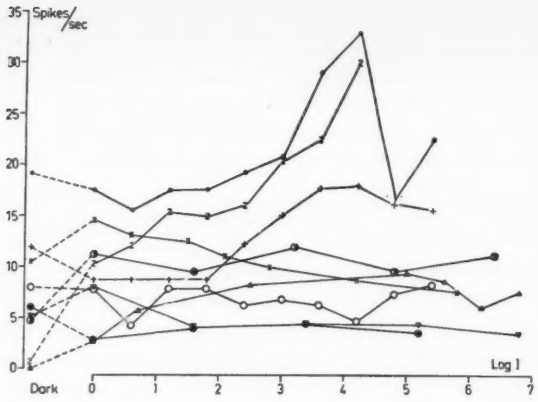

Fig. 5. Alteration in average rate of discharge with light intensity. Responses of 9 representative cells. Ordinate, rate of discharge, spikes/sec. Abscissa, light intensity, log units above lowest intensity at which a response was noticeable. Stimulus, slow flicker. Note rate of firing only increases slightly with intensity.

SÖDERBERG 1959). This is especially true if the resting discharge rate is high, but in most cells, the average firing rate during a flicker is little affected by the light intensity. This is shown in Fig. 5, where the results of 9 experiments are plotted together. In only 3 of the experiments is there a significant increase in the discharge rate as the light intensity increases. Only in one is this increase systematic. The geniculate input does, according to previous investigations, increase with light intensity but this is not reflected in these results. The light, however, had some effect on these cells, since the discharges were synchronised with the stimulus (see below).

Alteration in pattern of discharge. As the light intensity increased, so the pattern of discharge altered. A simple prolongation of discharge was rarely observed. Usually the impulses were redistributed in time, as though the resting discharge was modulated by an input both excitatory and inhibitory. This redistribution might occur in various ways.

In cells where there was a high resting activity, a weak flickering light caused little alteration in the discharge: if many traces were superimposed, it could be seen that there was a slight crowding of spikes at some period after the stimulus, but the 'resting activity' obscured the response. When a higher intensity was used, the response became more obvious, not only because more spikes congregated in one part of the trace, but, more strikingly, no other spikes occurred at any other time: the response fell on a silent background.

If a cell's resting discharge was sparse, the alteration in activity as the intensity was increased followed a different pattern, which is shown in Fig. 6.

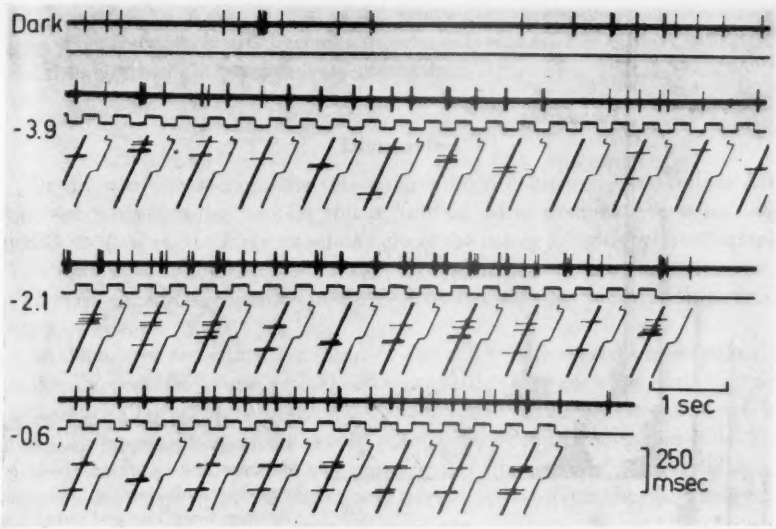

Fig. 6. Alteration in discharge with light intensity. Spikes recorded simultaneously by stationary spot and light-triggered sweeps. Note resting discharge consists of high frequency bursts. *Intensity —3.9.* The cell fires more often than in the dark, and in rhythm with the light but the latency is inconstant (see sweeps). *Intensity —2.1.* Latency is more constant, but successive responses do not resemble each other. *Intensity —0.6.* Successive responses are extremely similar. Note decrease in number of spikes per flash.

In response to a low intensity flicker, the average rate of discharge is increased so that the cell must be excited by the light. The continuous record shows that the cell responds to almost every flash with a burst similar to the resting discharge, but the form of the response and the latency is variable, as demonstrated by the light-triggered sweeps. When the light intensity is increased, the response becomes more 'regular', though the spikes per flash decrease, *i. e.* the total net excitation produced by light decreases. The change in response to light can only be described by saying that consecutive responses resemble each other more and more as the intensity of light increases. No other characteristic of the discharge alters systematically with the light intensity.

The experiment of Fig. 7 further analyses this point. All the intervals between the spikes discharged by a cell in the dark and in flickering light of various intensities, were measured, and plotted as histograms, in which the ordinate is the relative probability of obtaining any interval and the abscissa the interval. Between 28 and 30 flashes were used in making each histogram. The cell responded at both the 'on' and 'off' of the light.

In the resting discharge there is a great predominance of short intervals,

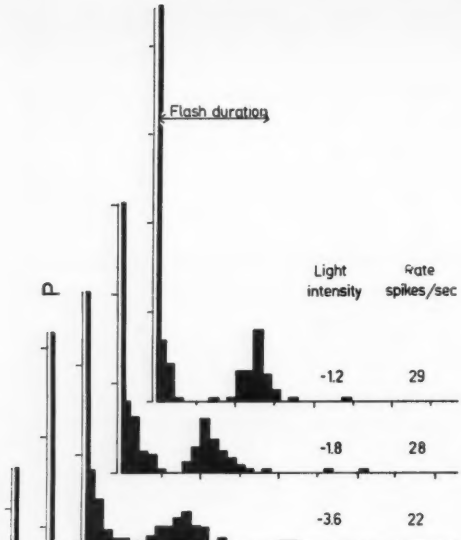

Fig. 7. Histograms showing the distribution of interspike intervals in darkness and flicker of various intensities: flash duration 140 msec. Average firing rate and light intensities shown. Abscissa, interval between successive spikes. Ordinate, relative probability of obtaining any interval. Note extreme preponderance of short intervals at all intensities, and the redistribution of longer intervals which occurs progressively as the intensity increases. For further description see text.

that is, the cell tends to fire in bursts. When the cell fires in response to the flickering light of intensity —4.3, the probability-distribution of intervals is much the same as in the dark, but there is a small increase in obtaining intervals of between 70 and 150 msec, *i. e.* approximately half the flicker period — the duration between 'on' and 'off' discharges. This hump becomes more pronounced as the light intensity increases, until at intensity —1.2 only short intervals and intervals of the approximate duration of the flash (*i. e.* the time between successive 'on' and 'off' responses) are obtained.

The average discharge-rate of the cell is little affected by the alteration in the light intensity but the distribution of intervals is greatly affected. Even at low light intensities, the 'bursts' are discharged in relationship to the flash (Fig. 6), and therefore, the initial part of each histogram represents the response to light (both 'on' and 'off' discharges). This scarcely alters till the highest intensity is reached; *i. e.* the number of spikes per evoked response and their frequency do not alter.

The alteration in distribution of the longer intervals implies that the interval between successive bursts becomes more constant, and the activity other than the response to light progressively diminished.

Discussion

In the previous section, the results have been compared with retinal data, but one must consider how far this is justified. Most mammalian retinal work has been done on the large ganglion cells of the cat — a highly selected sample. Furthermore although a wide variety of responses has been encountered in this work, the micro-electrode in the geniculate body may pick out only certain types of cell.

In default of recording the input of the cell whose response one measures, it is only possible to use retinal data generally. Recent work with differing techniques (THOMSON 1953, LENNOX 1958, 1959) suggests that earlier work is broadly representative of the whole population of retinal ganglion cells. It is probably safe to assume that the number and frequency of the retinal spikes broadly increases with intensity, and mechanisms of precise alternation of inhibition and excitation determine the flicker pattern. Unless the precision of the retinal response is not to be lost, either the geniculate must relay the optic nerve impulses very faithfully, or there must be some new transformation.

However the geniculate resting discharge alone makes it most unlikely that the geniculate synapses can act as simple one-to-one relays, since high discharge frequencies are only produced by retinal neurones when they are subjected to intense light, while in the geniculate cells, similar high frequency discharges are evoked both in the dark, and by lights of widely differing intensity. Accordingly, there must be reorganisation of afferent information in the geniculate, and the transformation of the pre- to the post-synaptic discharge pattern can be studied, though indirectly, by analysing the different modes of response to light.

If a geniculate cell is only excited and not inhibited by the afferent retinal discharge, its response would be like that of Fig. 1. The cell may follow rhythmic high frequency flicker but at lower rates, the successive responses become blurred. Under these conditions (low repetition rate, high light intensity) the retinal response is prolonged and so too is the discharge in the geniculate cell. Here however, the inhibitory mechanism which separates the retinal discharges is lacking. This type of response is rare, and most geniculate cells show evidence of both excitation and inhibition. To some extent, the retinal flicker mechanisms are reformed.

The frequency with which inhibitory phenomena are encountered in the geniculate is very striking. Indeed some cells are only inhibited by light, as in Fig. 2. This leads to responses which are very puzzling to analyse, since a

cell which is rhythmically inhibited by light may still appear to respond to flicker with what remains of its resting discharge. If the resting activity is sparse, there will be no fixed 'latency' of the response, as Fig. 3 demonstrates. The responses are further complicated by the fact that the background activity which is rhythmically inhibited is not the same as the resting activity (ARDEN and LIU 1960). Cells which respond in this way will also not follow slow flickers (Fig. 3 and 4) since the 'response' merges into the resting discharge of which it is a part. Neither can strongly inhibited cells flicker to any great rate. A maximal flicker rate of 12/sec was common.

In most cells both inhibitory and excitatory processes were observed. This is neurophysiological evidence of convergence of optic nerve fibres on geniculate cells. It might here be pointed out that the geniculate cells are affected by other neural paths than the optic tract (ARDEN and SÖDERBERG 1959). These have not been considered in the present paper.

Effect of intensity. In most geniculate cells it is evident that the initial frequency of discharge is of little importance. Another variable, the number of spikes discharged, only occasionally varies with intensity, and then the variation is not great. On the whole, geniculate responses are shorter than retinal ones.

On the other hand, the temporal pattern of the discharge does vary systematically as light intensity increases. The change can be described by saying that as the light intensity increases, each response becomes more like the preceding one, better synchronised with the flickering stimulus, and less like the resting discharge.

This, though different from the findings in the retina, is consistent with much work on the higher sensory pathways (AMASSIAN and HARDRESS 1958, JUNG 1958, MOUNTCASTLE, DAVIES and BERMAN 1957, ERULKAR and FILLENZ 1958). It is possible to appreciate, though only very generally how the change from the retinal frequency mechanism is transformed into the geniculate 'pattern' mechanism.

The geniculate cells have a pronounced tendency to fire in bursts, and fire even if the optic tract is interrupted. There are therefore other excitatory afferents, which act, during a flicker, as 'noise'. The activity in the geniculate cells is therefore only modulated by the activity in the optic tract. The increased effectiveness of the modulation as the light intensity increases, implies an increase in the excitation produced by the optic tract fibres. Since no, or only a few more spikes are produced by the geniculate cell, the excitation must be cut short by inhibition. Fig. 7 demonstrates that the pauses which follow the response to light (long intervals) are not due to the intrinsic properties of the geniculate neurone, since they do not occur in the same distribution when the cell is firing without any light stimulus.

Unless the technique employed excludes a population of cells which respond very differently, the responses must transmit information about stimulus inten-

sity. This would be possible if there was a cortical mechanism which responded to the periodicity of the discharges. Such a mechanism would have to function in an elaborate way (BULLOCK and DIECKE 1956) and does not seem likely.

It is very probable that, in a visual system such as the rabbit's, many cells act together as a functional unit. If a number of geniculate neurones act together in transmitting information one may say as a first approximation, that the consecutive responses of one cell may be taken as equivalent to the simultaneous responses of the whole group. In that case the maximal instantaneous frequency of discharge will increase, for the group, as the intensity rises, and the change will be very marked (Fig. 6). The latencies of different cells are long and variable near threshold and tend to shorter, fixed values as intensity increases (ARDEN and LIU, unpublished). Therefore such a multi-neuronal mechanism would operate better even than Fig. 6 suggests and will also minimise the effect of irregularities in the resting activity in the single cell.

The rabbit's visual system may be adapted to deal with moving stimuli only. It was partly to allow for this reason that a flickering stimulus was chosen to investigate the change in discharge with intensity. The flicker will mimic the rapid changes in intensity which occur on the retina, but under the experimental conditions all parts of the retina will be illuminated simultaneously, and the discharge of the geniculate cell may be very different if its retinal receptive field is traversed by a light. Cortical cells which only respond to the movement of a small light-source across the visual field have been described (HUBEL 1958).

Other possibilities, which seem less likely are

i) that the visual cortex in the rabbit is not the final analyser of light, and therefore the signals which it receives from the geniculate are not intimately connected with the parameters of the stimulus — this is possible since the decorticate rabbit suffers little visual defect (MARQUIS 1932).

ii) The intensity is signalled by recruitment. Little recruitment can be shown to occur in the lateral geniculate and would not be expected in the visual system where acuity increases with intensity.

iii) The behaviour of the cells is modified by the experimental procedures (anaesthetics, anoxia due to fall in blood pressure, or by the electrode). This is unlikely, since similar burst discharges have been frequently reported, and the animals and individual cells seemed to be in good condition.

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Acta physiol. scand. 1960. 48. 63—70

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Cholinesterases and Innervation of Skeletal Muscles

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Abstract

HÄGGQVIST, G. *Cholinesterases and innervation of skeletal muscles.* Acta physiol. scand. 1960. *48.* 63—70. — This investigation is directed to the possibility of a relationship between the behaviour of "specific cholinesterase" (AcChE), "non-specific butyrylcholinesterase" (BuChE), the two types of nerve endings known to occur in muscles and the number of phasic and tonic fibres in muscle nerves. It has been shown that muscles predominantly innervated by delicate tonic fibres have endings of a "grappe type" and stain heavier for BuChE. Muscles innervated principally by coarse fibres with endings of a "plaque type" stain for AcChE. Muscles innervated by mixed coarse and thin fibres possess both kinds of endings and show centres for AcChE as well as BuChE. It seems probable that the "en plaque" endings of the phasic fibres contain AcChE, whereas the "en grappe" endings of the tonic nerves, contain BuChE.

HOLMSTEDT (1957 a, 1958 b) found in M. biceps femoris from cats motor endings containing butyrylcholinesterase (BuChE) as well as acetylcholinesterase (AcChE). He employed a modification of Koelle's thiocholine method.

It has been known for a long time that in skeletal muscle there are two types of motor endings. One is characterized as "terminaisons en plaque" and belongs to the coarse, phasic nerve fibres. The other was first described by TSCHIRIEW (1879), who called them "terminaisons en grappe" and considered them to be embryonal motor end-plates. HÄGGQVIST showed (1938 and later) that they constitute the termination of fine efferent fibres, which he considered to be the nervous substrate for muscle tonus. The presence of a fine-fibered, tonic muscle innervation has since been confirmed, among others, by KUFFLER (1946), KUFFLER and GERARD (1947), KUFFLER and

HUNT (1949), BRECHT and FENEIS (1951) for frogs, KRÜGER (1957, 1958), KIRSCHE (1951) and GRANIT, HENATSCH and STEG for cats (1956).

The question arises whether any relation exists between the two cholinesterases, the two types of end-plates and phasic and tonic nerves.

Material and Methods

The examination of tonic muscles seemed ideal for study of this problem. HÄGGQVIST (1938) found N. facialis in Rhesus to be of markedly fine fibre size and in some facial muscles found only grape-like endings. In this animal, the same observations were made in M. sphincter ani, the classical example of a tonic muscle, and in its innervation. In contrast, N. abducens was composed predominantly of coarse fibres, and in M. rectus oculi lateralis could be found only "terminaisons en plaque". The examination has been made on *Gynomolgus*. This is closely akin to *Rhesus* and has been found to possess identical nerve patterns.

For staining HOLMSTEDT's (1957 a, b) modification of Koelle's thiocholine method was used. Whole muscles were stained embedded in paraffin and sectioned. Sometimes the reagent did not fully penetrate the muscles and this made the results unreliable. Therefore, the muscles were sectioned in a frozen state at 15 μ. These sections were stained. The animals were sacrificed with nembutal or ether, and the muscles excised within one hour. The muscles not immediately used were refrigerated at a temperature of — 40° C.

The time of incubation for AcChE was 5—15 min, but was extended to 3/4—2 hours for BuChE. The same times were employed for controls. A number of experiments were made on each muscle. An entire muscle has, in some cases, been employed for each combination in the series of experiments. In other cases, the muscle was divided in the direction of the fibres, into 2 or 4 pieces which were then employed for the different tests.

HOLMSTEDT recommends as an inhibitor of AcChE dimethobromide of 1: 5 bis (p-N-allyl-N-methylaminophenyl)pentan-3-one (BW) in a concentration of 5×10^{-6}. For BuChE he recommends Mipafox (M) in a concentration of 4×10^{-5}. The experiments were performed at a pH of 6—6.5.

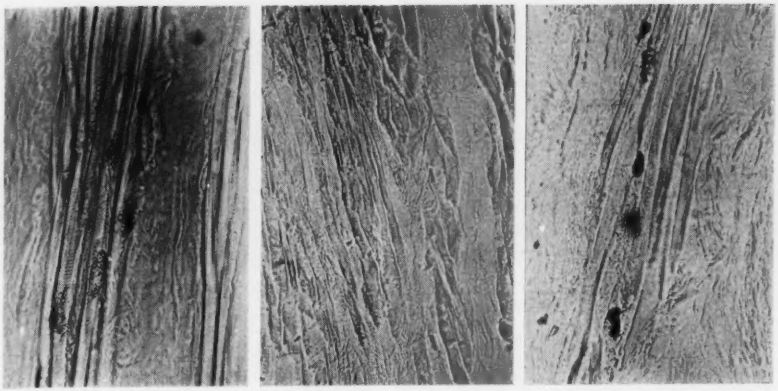
According to HOLMSTEDT, a complete series of examinations consists of seven combinations of substrate and inhibitor in accordance with the following schedule:

Slide	Treatment		ChE[2] visualized
	Inhibitor	Substrate	
A.....	—	AcThCh[1]	AcChE + BuChE
B.....	M	AcThCh	AcChE
C.....	BW	AcThCh	BuChE
D.....	M + BW	AcThCh	blank
E.....	—	BuThCh[1]	BuChE
F.....	M	BuThCh	blank
G.....	BW	BuThCh	BuChE

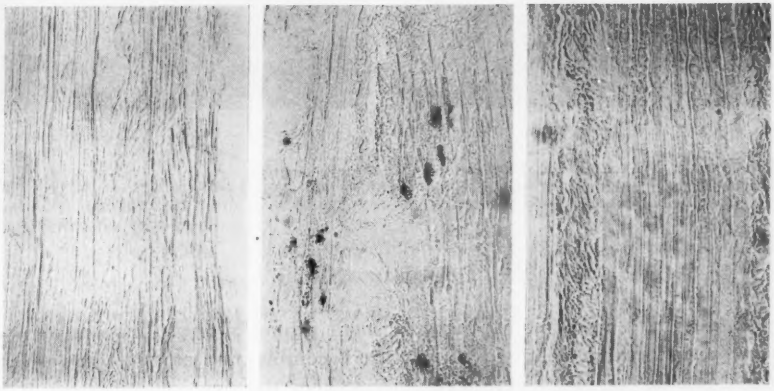
[1] = acetylthiocholine resp. butyrylthiocholine. [2] = Cholinesterase

After the above method has been tried, HOLMSTEDT considers the combinations B, D, F and G sufficient for routine use, and complete as well as incomplete series were employed in the experiments.

Results



A B C



D E F

Fig. 1. M. occipitalis, Rhesus.
A: positive; which indicates that endings with either AcChE or BuChE or with both exist in the muscle.
B: as a rule, negative. (See note 1.)
C: positive; thus, endings with BuChE exist.
D: is a control test where AcChE as well as BuChE are inhibited.
E: positive; presence of BuChE.
F: negative; due to Mipafox having inhibited BuChE.
G: positive; further support for the presence of endings with BuChE.

G

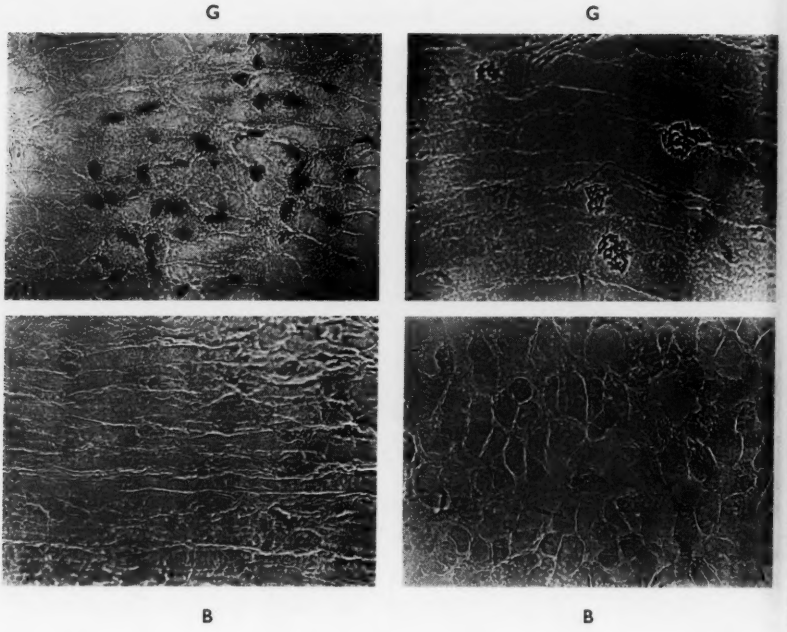


Fig. 2. Left: M. buccinator. B. negative, G. strongly positive.
Right: M. sphincter ani. B. negative, G. positive. (See note 2.)

Note 1. In a series of experiments small groups of crystallizations of copperthiocholinesulphate were found in the B combination on two slides which reminded one of motor end-plates but without their typical structure. It is possible that some endings with AcChE exist, but it is also possible that somehow an artefact has arisen.

Note 2. In one case endings with AcChE were found at the periphery of a muscle section. The muscle, however, lies with its dorsal part immediately in contact with the ventral caudal muscles and the possibility cannot for certain be precluded that isolated bundles of such muscles have been included when preparing the muscle.

Note 3. Diffuse precipitates in the connective tissues collaterally with reliable end-plate reactions are often found in the muscles of the eye if the sections are incubated more than 5—10 min. This probably is due to the great number of nerves. In the G combination typical end-plates have not been found but only a slight diffuse crystallization (see Fig. 3 G).

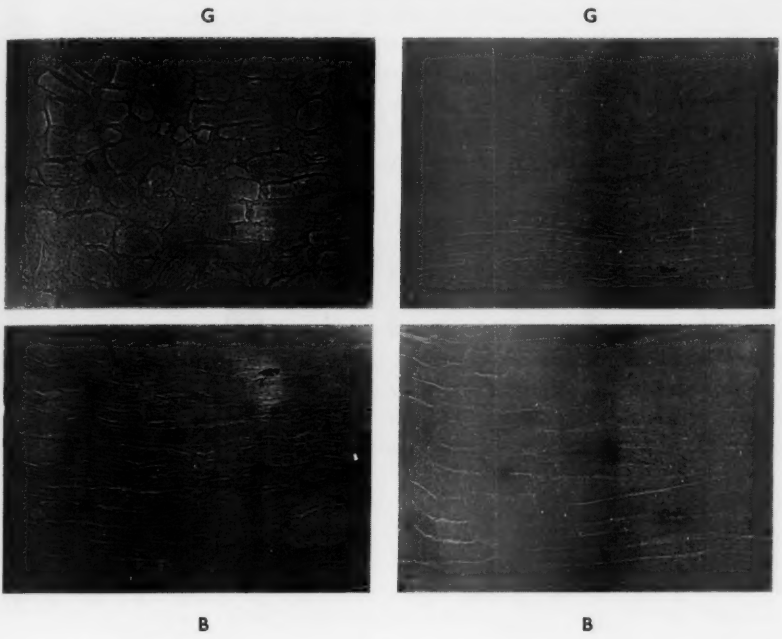


Fig. 3. Left: M. rectus oculi lat. B. positive, G. negative. (See note 3.)
Right: M. obliquus oculi sup. B. strongly positive, G. positive, but with considerably fewer reaction centres than in B.

Discussion

In all nerves, including pure efferents, there exists a mixture of coarse and fine fibres. There are great differences between nerves in the number of fibres belonging to either category. In N. facialis (Fig. 4) from *Rhesus*, HÄGGQVIST (1938) observed that fibers with a diameter below 5 μ constituted 70 %. On silver impregnation of some facial muscles he found only "terminaisons en grappe".

Some fibres, however, have a diameter of up to 8—9 μ. Such fibres probably belong to the phasic system and it is therefore likely that there also exist "terminaisons en plaque" in some facial muscles. As regards these coarse fibres there are two possibilities. They can be homogeneously distributed among different muscles or they can appear in certain definite ones, whereas other facial muscles entirely lack phasic innervation. The latter seems to me more likely. M. fronto-occipitalis is a muscle which often contracts voluntarily and consequently it should not be surprising that endings with AcChE are to be found there. This thin muscle is, however, extensive in monkeys, very pale and difficult to distinguish from connective tissue; consequently certain

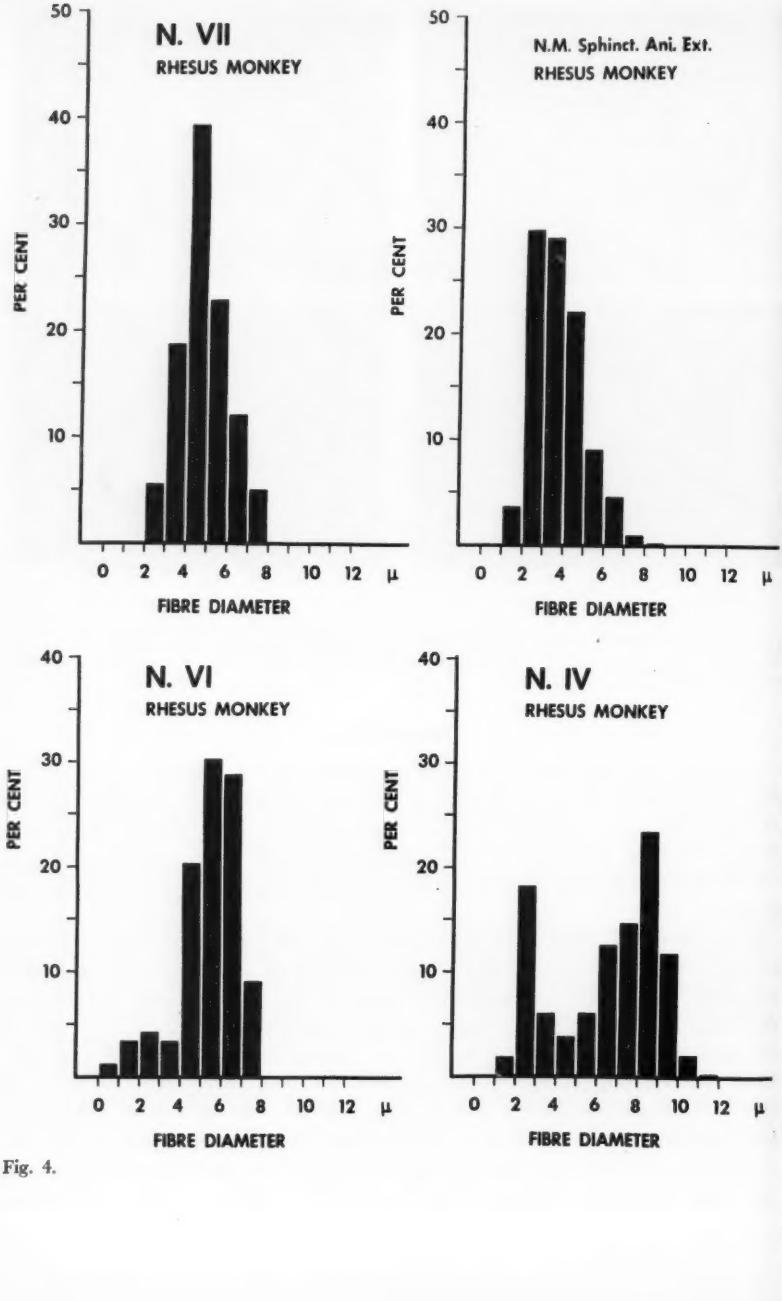

N. VII
RHESUS MONKEY
N.M. Sphinct. Ani. Ext.
RHESUS MONKEY
N. VI
RHESUS MONKEY
N. IV
RHESUS MONKEY
PER CENT
FIBRE DIAMETER
0 2 4 6 8 10 12 μ
10 20 30 40 50

Fig. 4.

pieces may have escaped attention in the preparation. As only two slides in one out of seven series show a positive reaction for AcChE the possibility exists that an artefact has appeared during the staining.

M. buccinator on the other hand is a typical tonic muscle that seldom contracts voluntarily. In five cases in which the entire muscle was examined not a single ending with AcChE was found, whereas endings with BuChE were found in large numbers.

In N. m. sphincter ani HÄGGQVIST (1938) found only 15.5 % of the fibres to be above 5 μ in diameter. In the examinations all fibres of the nerve were measured (Fig. 4). Some fibres were 8—9 μ in diameter. In this case the nerve is mixed and it is probable that some — possibly all — of the coarse fibres are proprioceptive. In a large number of muscles impregnated with silver and cut serially, only grape-like endings could be observed. It is, therefore, of interest to establish that in this case, in all the experiments except one, only endings with BuChE have been observed. In the exceptional case there exists the possibility of bundles of ventral caudal muscles having been included along with the others.

M. rectus oculi lateralis receives its nerve fibres through the VIth cranial nerve, N. abducens. This nerve (Fig. 4) is in direct contrast to the former ones, HÄGGQVIST (1938) finding a diameter below 5 μ in only 8.8 % of the fibres. On silver impregnation of the muscle he found only "terminaisons en plaque".

The cholinesterase experiments reported above show that a large number of endings with AcChE exist in the muscle. On the other hand none with BuChE have been established. A faint diffuse precipitate of BuChE has been observed, but no decision could be made as to the reacting structure.

M. obliquus oculi superior is somewhat intermediate to the muscles mentioned above. It is innervated by N. trochlearis, the IVth cranial nerve. In his examinations HÄGGQVIST (1938, Fig. 4) found that the calibre diagram of this nerve showed two peaks, one at 2—3 μ and the other at 8—9 μ. 29.7 % of the fibres were below 5 μ. The coarsest fibres measured 11—12 μ. He found both kinds of endings in the muscle.

In the ChE experiments reported above, end-plates with AcChE as well as BuChE have been found. The former are far more frequent.

Thus, there seems to be good parallelism in the relationship between coarse and fine nerve fibres in the muscle nerves and endings of a plaque type and a grappe type respectively on one hand and the existence of endings with AcChE and BuChE on the other. The former endings are of considerably larger dimensions than the latter. In this connection it may also be of interest to note that in order to obtain a satisfactory stain the incubation time for BuChE must be made several times longer than for AcChE. This may be due to the amount of ChE in the isolated endings.

I wish to thank Dr. HOLMSTEDT for advice and information concerning his technique. Professors C. G. BERNHARD and S. GARD furnished monkey material and Miss RAGNA HÄGGQVIST assisted with the technical work.

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Lipoprotein Lipase Activity in Adipose Tissue from Hen, Chicken and Adult Human

By

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Abstract

ANGERVALL, G. *Lipoprotein lipase activity in adipose tissue from hen, chicken and adult human.* Acta physiol. scand. 1960. *48.* 71—77. — Lipoprotein lipase was extracted from acetone powder of hen and chicken adipose tissue with dilute ammonia. The enzyme was tested in vitro in a system with milk cream suspension as a substrate and pure albumin or inhibitor-freed plasma as a fatty acid acceptor. The activity was measured as a decrease in optical density in the system and as a release of unesterified fatty acids per 10 mg of enzyme extract. Lipoprotein lipase activity was three or four times greater in extracts of chicken adipose tissue as compared with those of hen. In adult human adipose tissue, however, there was no or insignificant lipoprotein lipase activity in three of four cases. — The role of inhibitors of lipoprotein lipase in connection with the method was discussed.

QUAGLIARELLO and SCOZ (1932) described an enzymatic splitting of triglycerides by an extract of adipose tissue of the dog. Lipolytic activity in adipose tissue in man was found by MARBLE and SMITH (1942). With histochemical technic GOMORI (1945, 1946) found lipolytic activity in adipose tissue from rabbit and rat and occasionally in human adipose tissue. RENOLD and MARBLE (1950) demonstrated lipolytic activity of an enzymatic nature in subcutaneous adipose tissue of both man and rat and this enzyme had many similarities with lipoprotein lipase (described by KORN 1954 and 1955). Lipoprotein lipase was found in rat, rabbit and calf heart and in rat and rabbit adipose tissue; in chicken adipose tissue KORN and QUIGLEY (1957) found a larger amount of the enzyme. The enzyme was called lipoprotein lipase because

it catalyses the hydrolysis of the triglyceride moiety of chylomicrons and beta-lipoproteins at a much faster rate than simple triglyceride emulsions.

Using the extraction procedure of KORN and QUIGLEY (1957), the present author found only insignificant lipoprotein lipase activity in human adipose tissue but a large activity in chicken adipose tissue (see Results). One of many possible explanations for this difference might be that in the case of human tissue the adipose tissue was taken from adults, and in the case of chicken tissue from a growing animal. This prompted the following investigation on the lipoprotein lipase activity of hen and chicken adipose tissue.

Material and methods

Enzyme preparation. Hen and chicken adipose tissue were obtained partly as frozen pooled fat from commercial sources, partly from individual hens and chickens, whose fat immediately after decapitation was frozen and stored at — 20° C. The hens were about 2—3 years old and the chickens about 6—7 months old. Because of the sparse adipose tissue in the six separate chickens, the fat from these was pooled (Ch × 6). This fat and the fat from Hen 10, which also had very sparse adipose tissue, was difficult to free from extraneous material. Adipose tissue from adult human was taken at surgery (two cases of hernia abdominalis, one case of varices cruris and one case of cancer mammae). The enzyme lipoprotein lipase was extracted from the tissue acetone powder with dilute ammonia according to KORN and QUIGLEY (1957).

Substrate. The substrate was a suspension of homogenized cream in physiological saline ($Cream_{NaCl}$)[1] activated with normal human plasma for 30 min at 38° C. The concentration of the cream suspension was chosen to give an optical density of about 1.4 in the clearing system (see below), that is a concentration of butter fat of about 100 mg per cent.

Clearing systems

1 ml LPL (lipoprotein lipase) = 10 mg of lyophilized acetone powder extract in one ml 0.025 M ammonia.
P_{oDP} = inhibitor-freed plasma (HOOD and ANGERVALL 1957).
Alb. = 10 per cent human albumin in saline. Albumin was 99—100 per cent electrophoretically pure (from KABI, Stockholm, Sweden).

Systems

A. 0.8 ml LPL + 0.4 ml 1 M $(H_4N)_2SO_4$ + 6.4 ml P_{oDP} + 0.4 ml $Cream_{NaCl}$
B. 1.0 ml LPL + 0.4 ml 1 M $(H_4N)_2SO_4$ + 8.0 ml P_{oDP} + 0.4 ml $Cream_{NaCl}$
C. 1.0 ml LPL + 0.2 ml 1 M $(H_4N)_2SO_4$ + 3.5 ml alb. + 0.3 ml $Cream_{NaCl}$
D. 1.0 ml LPL + 0.2 ml 1 M $(H_4N)_2SO_4$ + 3.5 ml alb. + 0.3 ml $Cream_{NaCl}$ + 0.5 ml saline or $5—10^{-2}$M sodium deoxycholate.

Incubation was made at 24° C or 37° C. Every incubation was made in duplicate tubes.

In system D (Pool Ch V and Pool Hen II) the amount of substrate ($Cream_{NaCl}$) was changed. In one set of duplicate tubes the concentration of $Cream_{NaCl}$ was chosen to give the usual suitable initial optical density of 1.4. In those tubes where the release of

[1] Much earlier work with this substrate and comparison with suspension of chylomicrons and various emulgations of vegetable oils, have shown this to be a most reliable substrate (ANGERVALL and HOOD 1957).

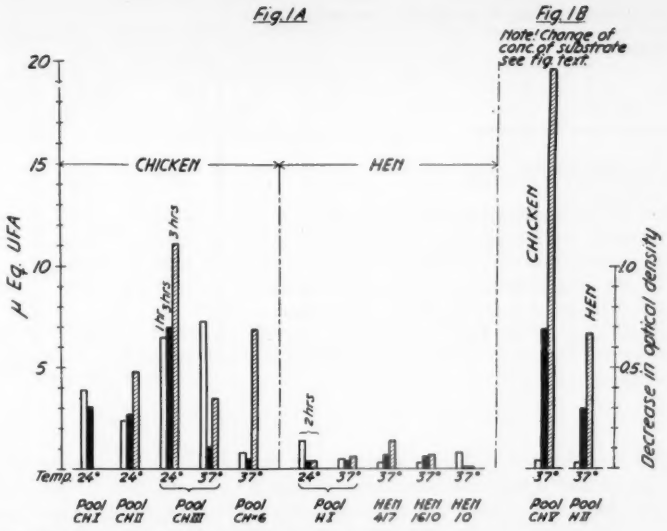

Fig. 1. *Lipoprotein lipase of chicken and hen adipose tissue.*
Incubation systems, see table and methods. Open staples represent decrease in optical density in the system in one hour. Filled staples represent LPL activity in 10 mg of enzyme extract, measured as UFA release in μeq in one hour. Hatched staples represent LPL activity measured as UFA release in μeq in one hour, related to 10 g of adipose tissue (wet weight). In all experiments in Fig. 1 A, the amount of substrate ($Cream_{NaCl}$) was chosen to give an optical density of 1.4 in the system. In the experiments in Fig. 1 B (pool Ch V and pool Hen II) the substrate in the open staples was unchanged, but in the filled and the hatched staples the substrate was 14 times greater than that in the other ones.

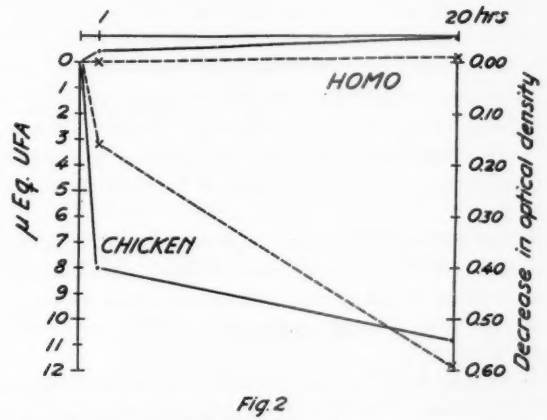

Fig. 2.

Fig. 2. *Lipoprotein lipase of chicken and human adipose tissue.*
Incubation system A (see methods). ——— = decrease in optical density in the system. — — — = LPL activity in 10 mg of enzyme extract measured as UFA release in μeq.

Table I. Lipoprotein lipase in chicken and hen adipose tissue

Adipose tissue	Chicken		
	Pool CH I	Pool CH II	Pool CH II
$\frac{\text{Weight of aceton powder}}{\text{Wet weight}} \times 100$	1.3	1.2	1.0
Dry weight of 2 ml aceton powder extract in mg	—	15.6	8.6
Incubation system and incubation temperature	A.24°	B.24°	C.24°
Decrease in optical density in one hour in the system	0.39	0.24	0.65
Release of UFA in μeq per 10 mg of extract in one hour	3.1	2.7	[1](7.0)
LPP activity — calculated per 10 mg of adipose tissue — as UFA release in μeq in one hour	—	4.8	[1](11.1)

[1] = incubation in 3 hrs

unesterified fatty acids (UFA) was measured, the amount of $Cream_{NaCl}$ was increased 14 times as compared with the earlier experiments.

Lipoprotein lipase activity was measured as the decrease in optical density in the system in one hour (Beckman B spectrophotometer, one cm pathway, at 650 mμ) and as release of unesterified fatty acids (UFA) in one hour. The determination of UFA was made according to the method described by GROSSMAN *et al.* (1954). The titration was made with 0.02 N NaOH with a modification (LØVTRUP and ROOS 1959) of a microburette, described by HEATLEY (1935). With the capillary used in this investigation 0.35 cm corresponds to 1 μl. Thymol blue, dissolved in methanol, was used as indicator. Nitrogen was bubbled through the alcohol solution during the titration. Standard deviation of 70 duplicate determinations was $\pm$ 0.032 meq/l.

Results

The results are given in Table I and Fig. 1. Clearing activity and the ability to set free UFA was at least 3—4 times greater in the extract from chicken adipose tissue than in that from the hen. The activity was greatly inhibited by sodium deoxycholate at a concentration of 5×10^{-3} (less than 1/3 UFA released in one hour).

The considerably increased activity in the experiment with the large substrate concentration (Ch V and H II) seems to show that the activities in the other experiments by no means give absolute measures of the enzymes present. They may, however, give a rough indication of differences between chicken and hen in this respect.

From the beginning, these experiments with chicken fat were performed as controls of investigations of the LPL activity in subcutaneous human adipose tissue, obtained at various surgical procedures. Fig. 2 shows such an experiment where a comparison was made between the LPL-activity in human and

		Hen					Chicken	Hen
Pool CH III	Pool CH×6	Pool H I	Pool H I	Hen 417	Hen 1 610	Hen 10	Pool CH V	Pool H II
2.5	8.1	2.1	1.3	1.4	1.4	5.2	2.1	1.9
12.6	19.0	10.7	13.2	14.9	8.2	11.2	13.7	12.2
C.37°	C.37°	C.24°	C.37°	C.37°	C.37°	C.37°	D.37°	D.37°
0.73	0.08	²0.14	0.05	0.03	0.03	0.08	0.04	0.03
1.1	0.5	²0.4	0.4	0.7	0.6	0.0	6.9	3.0
3.5	6.9	²0.4	0.6	1.4	0.7	0	19.7	6.9

² = incubation in 2 hrs

chicken adipose tissue (Chicken-Pool Ch I as in Table I and Fig. 1; system A, temperature 24°). Another three experiments have been made on human adipose tissue. All but one (1.6 μeq per 10 mg of extract in one hour) showed no or insignificant LPL activity.

The earlier demonstration of the inhibition present in normal human plasma (ANGERVALL and HOOD 1957) raised the question, whether the amount of blood present in adipose tissue from human adult might have any influence. When 10 ml human plasma was mixed with 100 g chicken adipose tissue (Pool Ch II) immediately before acetone extraction the acetone powder weight was 2.2 per cent of the wet weight. When the ammonia extract was incubated according to system B 24°, the decrease in optical density in one hour was 0.05, and the release of UFA per 10 mg of extract was 0.6 μeq in one hour. The corresponding values without adding plasma (see Table I, Pool Ch II) was 1.2 per cent, 0.24 and 2.7 μeq respectively.

Because of these and other difficulties (see Discussion), preliminary experiments have been done according to GORDON and CHERKES (1958) but using slices of frozen adipose tissue instead of thin leaves of epididymal fat. In one such experiment with chicken fat (Ch II), there was a LPL activity corresponding to a release of 14 μeq per 10 g of adipose tissue in one hour, as compared with 4.8 μeq with extraction procedure of KORN and QUIGLEY (1957).

Discussion

In this investigation there was greater LPL activity in the extract of chicken than in that of hen, both when the activity was related to the amount of extract and to the amount of adipose tissue. This has been shown with adipose tissue from two different commercial sources (pooled fat) and from chickens and

hens from the same family (Ch × 6 and Hen 417, 1610 and 10). GORDON and CHERKES (1958) and RESHEF, SHAFRIR and SHAPIRO (1958), who investigated the release of UFA from thin leaves of depot fat (epididymal and mesenterial respectively) in vitro in a medium with 5 per cent albumin showed that the release of UFA was more rapid when the fat was taken from fasting animals than from fed ones. In this investigation the animals Ch × 6, Hen 417, 1610 and 10 were fasting for about 20 hours before decapitation. As to the fat from commercial sources there is nothing known whether there were any differences in hen and chicken in this respect, but it is not probable.

Especially the poor results as to the LPL-activity in human adipose tissue must be judged as to the occurrence of LPL-inhibitors in the system. The occurrence of inhibitors of LPL a) in plasma of dog has been shown by HOLLETT and MENG (1956) and in that of human by ANGERVALL and HOOD (1957), b) in white blood cells and platelets components (FEKETE, LEVER and KLEIN, 1958); in platelets also of MITCHELL (1959), c) in tissue extracts from rabbits and human autopsies with the highest inhibitory activities in liver, spleen and kidney but low in skin, brain and adipose tissues (KLEIN, LEVER and FEKETE 1958). The latter authors did not discuss the possibility of any relation between the inhibition and the amount of blood in the organs. With a saline extract of adipose tissue they got 24 per cent inhibition of a clearing system. When the author added plasma to chicken adipose tissue before extraction, the LPL-activity of the extract, measured as decrease in optical density in a clearing system, was only 21 per cent of the activity of the extract of chicken adipose tissue alone. As to the release of UFA in the same system the inhibition was 22 per cent calculated upon 10 mg of extract.

As to the content of blood in hen and chicken adipose tissue, there was no difference in the macroscopic appearance or in the extracts, but in the case of human adipose tissue there was macroscopic blood present in spite of repeated washing and the extracts were dark brownish discoloured. There is a possibility that the occurrence of blood in the human adipose tissue was the cause of the low or absent LPL-activity in the enzyme extracts.

When extracts made according to KORN and QUIGLEY (1957) from chicken and hen adipose tissue were put into a clearing system with postheparin plasma, there was an inhibition of clearing. Thus, the extract should contain inhibitors as well as LPL. ANGERVALL and HOOD (1957) prepared the inhibitors of plasma in the same manner as they prepared clearing factor from postheparin plasma. They also adsorbed clearing factor as well as its inhibitors on Ba SO_4, and both were eluated with 0.1 M citrate. Thus LPL and its inhibitors follow each other closely.

Preliminary experiments with direct incubation of slices of adipose tissue according to GORDON and CHERKES (1958) seemed to give higher LPL-activity than the extraction procedure according to KORN and QUIGLEY (1957).

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Retention of Particles by the Reticulo-Endothelial System

By

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Abstract

JUHLIN, L. *Retention of particles by the reticulo-endothelial system.* Acta physiol. scand. 1960. *48.* 78—87. — Spherical particles of methyl methacrylate were injected intravenously in rabbits and mice. They are taken up by the R. E. S. primarily in the liver, spleen and bone marrow. The elimination from these organs was then studied, in some cases up to 18 months after injection. Three types of particles were used: Positively charged particles 0.05 μ in diameter and negatively charged particles 0.1 μ and 0.8 μ in diameter. These particles are all taken up by the R. E. S. and eliminated in a similar fashion. Approximately 50 per cent of the injected dose is eliminated from the body during the first month. In mice after 18 months about 10 per cent remained in the total body. Special polynuclear histiocytic cells were then found in the liver in which 0.8 μ particles could be seen with ordinary microscopy. The 0.8 μ particles increased in the spleen of rabbits and mice and in the bone marrow of rabbits during the first weeks. This seemed to be the result of a liberation of particles from the liver to the blood stream. The total amount of femur-equivalent bone marrow in the rabbit skeleton was found to be 1.5 per cent of the body weight. The distribution of particles in different bones showed a bilateral symmetry, was not influenced by dose or particle size and was the same 3 hours and 20 days after injection.

Intravenously injected particulate matter is removed from the blood stream by the reticulo-endothelial (RE-)cells of the liver, spleen, bone marrow and lungs. When particles of thorium dioxide (Thorotrast®) are used they have been found to remain in the RE-cells several years after injection (for ref. see STENSTRÖM 1941, REICHARD and GORDON 1956). HURCH *et al.* (1957) investigated thoroughly the excretion of thorium and its daughter products in two patients. Less than 1 % of the particulate matter administered was excreted during the first month, and a biologic half-life of more than 400 years

was calculated. REEVES and MORGAN (1937) measured the gamma radiation and found that about 80 % of the injected dose was retained in the liver and spleen in man after 4 years. EASTON (1952), however, found that only as much as about 1/3—1/2 of the injected dose was retained in the RE-cells after a year. He found that the loss of Thorotrast occurred principally during the first 10 days after injection.

The amount of Thorotrast was estimated by measuring its radioactivity. The decay curve for Thorotrast is rather complicated since after some time the Thorotrast particle contains, α, β and γ emitters. The calculations are further complicated by the fact that some of the daughter products are not insoluble but are dialysable. The particles vary greatly in size, which might partly explain the divergent results. Incineration also often introduces a large error in the analyses (HALE 1953).

Particles of radioactive $CrPO_4$ 2—4 μ in diameter were used for similar studies by MCCORMICK *et al.* (1954). They found in some experiments that the particles were retained in the liver and spleen during 12 weeks after injection.

A more suitable method for studies on these and related problems of the R. E. S. function should be the use of the strongly fluorescent spherical particles of methyl methacrylate by JUHLIN (1956). Both smaller and larger particles with different surface properties were prepared in batches with rather uniform size. The present study reports the effect of size, surface charges and doses of the particles on their elimination from the body.

Methods

Particles. The particles were prepared by emulsion polymerisation of methyl methacrylate and marked with a fluorescent colour as described earlier (JUHLIN 1956).

The following hydrophilic particles were used:

1. Negatively charged particles with a mean diameter of 0.1 μ (= Sh, JUHLIN 1956).

2. Negatively charged particles with a mean diameter of 0.8 μ (= Lh, JUHLIN 1956).

3. Positively charged particles with a mean diameter of 0.05 μ. These particles were prepared with a cationic emulgator (octadecyldimethylaminoacetate) in the following way: 4 g octadecyldimethylaminoacetate was dissolved in 14.5 g of water during slight warming whereafter 360 g distilled water and 1 ml 30 % hydrogen peroxide H_2O_2 were added. Fluorescent Green (0.25 g) was dissolved separately in 125 g methyl methacrylate and added to the solution prepared earlier. Thereafter all was polymerized under agitation at about 80° during 40 min. During polymerisation part of the dye fell out so the fluorescent-dye content was only about 1/10 that of the particles prepared earlier (under 1 and 2).

Estimation of particle charge

To 10 ml of a particle suspension containing 2.4 % by weight of solids 2 ml of fresh heparinized rabbit plasma was added; 1 ml of this suspension was then added to 10 ml of 0.2 M acetic acid buffer of pH 5.4 and placed in a U-shaped tube for free electro-

Table I

Rabbit no.	Injected dose of particles in mg	Time after inj.	Calculated amount of particles in mg/gram dried liver	
			Fluorescence determination	Methyl methacrylate determination
I	192	3 hours	8.3	6.1
II.....	480	2 months	4.2	2.8
III	192	2 months	2.9	0.5
IV	192	2 months	3.0	0.6

phoresis against the same buffer. A voltage of 440 V was used, which gave about 20 V/cm. The suspension phase with negatively charged particles migrated towards the anode at a speed of about 4 mm/min, whereas by a similar procedure the suspension containing positively charged particles migrated towards the cathode at about the same speed.

A very rough estimation of the charge on the surface of each particle can then be calculated according to the formula given by ALEXANDER and JOHNSSON (1949), $\frac{V}{x} = \frac{Q}{4\pi\mu a} \cdot \frac{f(Ka)}{1+Ka}$ where Q = net charge, μ = viscosity of suspending medium, $f(Ka)$ = the Henry factor, V = velocity of migration, x = potential gradient, and a = radius of particle. These give $Q \cong 10{,}000$ negative charges per 0.1 μ particle and $Q \cong 2{,}500$ positive charges on the 0.05 μ particle.

Experimental procedure

Rabbits. Male albino rabbits weighing about 2 kg were used. They were fed on hay, oats, and water ad lib. The rabbits were given intravenous injections in the ear 2 times with an interval of one hour. Altogether 600 mg of the 0.8 μ particles were administered as a 3 % suspension in 5 % sterile glucose. The 0.1 μ particles were injected in total dose of both 192 mg and 480 mg. The suspension contained 1.7 and 4 % of solids in 5 % sterile glucose. After certain intervals the rabbits were killed and the amount of particles estimated by measuring their fluorescence in the liver, spleen, lung and bone marrow as described earlier (JUHLIN 1958).

Mice. White mice of a commercial strain (20—25 g) given food and water ad lib. were used. Particles of 0.05 μ and 0.1 μ diameter were given intravenously in the tail vein in a dose of 12 mg of solid particles suspended in 0.2 ml of sterile glucose. Particles of 0.8 μ diameter were given in a dose of 9 mg of solid particles in 0.3 ml of sterile glucose. At certain intervals thereafter the retained particles were estimated by measuring the fluorescence in the liver, spleen and lung.

Control determinations of plastic in the liver

To ascertain that the fluorescent dye really is bound in the plastic particles the amount of methyl methacrylate was determined in certain experiments. Rabbits received intravenous injections of negatively charged 0.1 μ particles and were killed after 3 hours and 2 months. Fluorescence determination of the particle content was performed on 1 g tissue taken from the liver. The rest of the liver was frozen-dried where-

Table II.

Particle size in μ	Dose in mg	Time after injection	Mg particles in total skeleton = A	Mg particles/g femur bone marrow = a	$\frac{A}{a}$	Body weight kg	Amount of femur-equivalent bone marrow in percent of body weight
0.1	192	3 hours	44.6	1.50	29.7	2.0	1.49
»	»	»	52.4	1.75	29.9	2.1	1.42
»	»	20 days	38.1	1.29	29.5	1.9	1.55
»	»	»	28.8	0.84	34.3	2.2	1.55
»	480	3 hours	103.2	4.10	25.2	2.0	1.26
»	»	»	113.4	4.06	27.9	1.8	1.55
0.8	600	»	74.2	2.40	30.9	2.2	1.40
»	»	»	60.0	1.90	31.6	2.0	1.58
»	»	20 days	104.8	3.13	33.5	2.4	1.40
»	»	»	144.0	4.45	32.4	2.0	1.62

by it decreased in weight by about 75 %. Two grams of the dried liver was extracted with hexane during 18 hours in a Soxhlet apparatus. This extraction removes the greater part of the fat in the liver. Subsequent extraction was done in acetone for 24 hours, and the extract was dried. The dry substance, with the plastic, was again treated with absolute alcohol in a Soxhlet apparatus during 6 hours for removal of the rest of the fat. The residue was weighed out as plastic. The results appear in Table I.

The amount of particles in the liver estimated by fluorescence determinations is higher than that obtained from methyl methacrylate determinations. The difference might be due to that part of the methyl methacrylate is lost during the extractions. A like particle concentration determination results, however, when the methacrylate method is corrected for this supposed loss (about 2 mg/g dried liver). At any rate, there is no evidence for diffusion of the fluorescent dye from the particles. A decrease in fluorescence therefore really means a decrease in the amount of plastic particles.

Estimation of particles in rabbit bone marrow

Soft tissues were thoroughly scraped away from all bones, which were then crushed by hand to expose the marrow. They were subsequently shaken in acetone for 12 hours. After centrifugation the fluorescence content was determined as described earlier (JUHLIN 1958). The amount of particles in the different bones could then be computed. Such total bone marrow determinations were carried out on 10 rabbits. The dose and size of the particles used appear in Table II where the results are given.

From the total amount of particles in the skeleton (A mg) and the amount of particles/g femur bone marrow (a mg) one obtains that the total skeleton contains A/a g femur-equivalent bone marrow. This was found to be 1.5 % of the body-weight. About the same per cent is found in the literature but there it is an anatomical and not a functional value.

This value (1.5 %) for the amount of femur-equivalent bone marrow will be used in the following to deduce the whole skeleton dose of particles from a femur analysis. Since no measurable quantities of bone marrow could be obtained in mice, the investigation was performed on rabbits only.

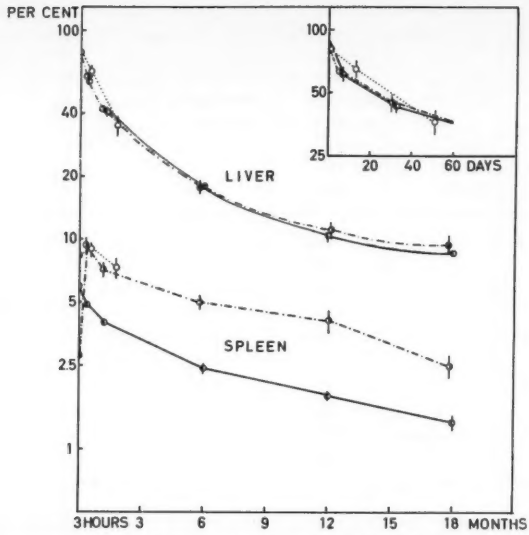

Fig. 1. Disappearance of particles in liver and spleen after a single intravenous injection in mice.
Abscissa: Time in months.
Ordinate: Amount of particles in per cent of injected dose. Zero time is 3 hours after injection.
Each point represents 5—8 mice. The vertical lines indicate the standard error of the mean.
◐——◐ Negatively charged 0.1 μ particles. Dose 12 mg/mouse.
◑·–·–·◑ Negatively charged 0.8 μ particles. Dose 9 mg/mouse.
○·····○ Positively charged 0.05 μ particles. Dose 12 mg/mouse.

Results

The results appear from Fig. 1—5. The following conclusions may be drawn:

1. In mice intravenously injected negatively and positively charged particles are taken up chiefly (85—90 %) by the RE-cells in the liver and spleen (Fig. 1). In rabbits about 25 per cent of injected dose of 0.1 μ particles and 10 per cent of 0.8 μ particles are found in the bone marrow 3 hours after injection (Fig. 2 b). In both animals about 50 per cent is eliminated from the body during the first month (Fig. 3). The mice were followed for 18 months, at which time about a 10 per cent total body retention of the injected dose was observed (Fig. 1).

2. Only 0.8 μ particles could be visualized with ordinary microscopy. The first days after injection they were found to be diffusely localized in the endothelial cells of the liver sinusoids. Six months after injection of the different types of particles histiocytic polynuclear cells appeared especially around the central veins of the liver lobules (Fig. 5). Particles of 0.8 μ size could be seen in these cells but are not visible in the printed reproduction. No signs of malignancy were found.

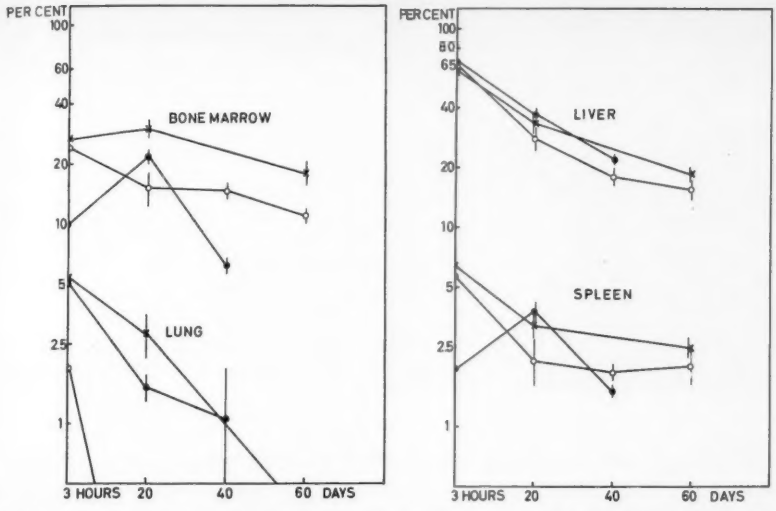

Fig. 2 a, b. Disappearance of particles in liver, spleen, bone marrow and lung after a single intravenous injection in rabbits.
Abscissa: Time after injection. Zero time is 3 hours after injection.
Ordinate: Amount of particles in per cent of injected dose.
Each point represents 5—8 rabbits. The vertical lines indicate the standard error of the mean.
○——○ Negatively charged 0.1 μ particles. Dose 192 mg/rabbit.
×——× Negatively charged 0.1 μ particles. Dose 480 mg/rabbit.
●——● Negatively charged 0.8 μ particles. Dose 600 mg/rabbit.

3. The amount of 0.8 μ particles in the spleen and bone marrow of rabbit was higher 20 days after injection than 3 hours after (Fig. 2 a, b). In mice there was a corresponding increase of 0.8 μ particles in the spleen during the first week (Fig. 1). A corresponding but smaller ($P < 0.05$) increase in the bone marrow is seen also with the higher dose of 0.1 μ in the rabbit.

4. The total amount of femur-equivalent bone marrow in the skeleton was found to be 1.5 % of the body weight. The percentual distribution of particles between different bones showed a bilateral symmetry and was not influenced by dose or particle size and was the same both 3 hours and 20 days after injection. In Fig. 4 the total amount of particles in all bone marrow has been equated to 100 per cent to show the percentual distribution between different groups of bones.

Discussion

It has previously been known that the negatively charged particles are taken up by the reticulo-endothelial system. JANSCÓ (1955) found that uncharged particles could be taken up in the reticulo-endothelial system. It has

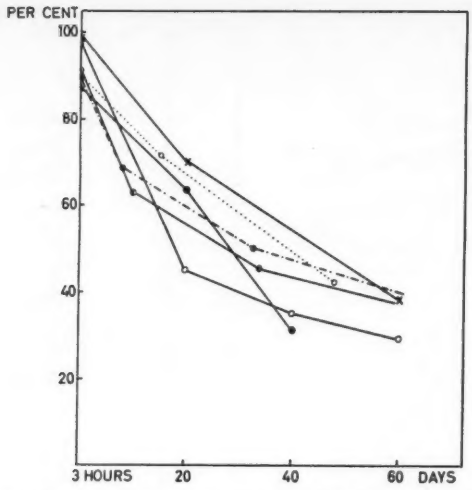

Fig. 3. Disappearance of particles from the total body of rabbits and mice. Particles are designated as in Fig. 1 and 2.
Abscissa: Time in days.
Ordinate: Amount of particles retained in the body in per cent of injected dose.

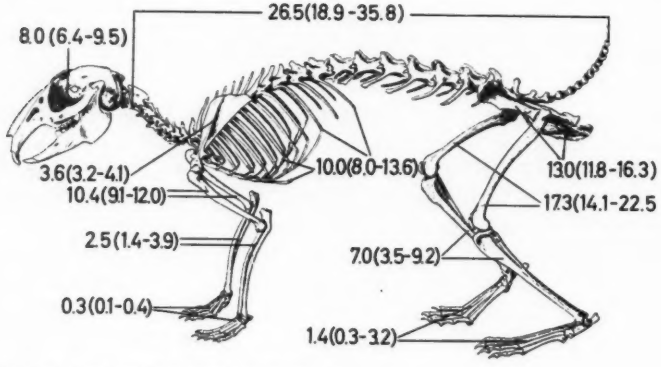

Fig. 4. Skeletal distribution of particles in rabbits.
The figures given indicate means and ranges in per cent of skeletal dose.

here been shown that also positively charged particles are taken up by the reticuloendothelial system. It would seem therefore that the electrostatic factor is of minor importance.

Unfortunately, the amount of fluorescence in our positively charged particles was too small to permit an accurate analysis of their disappearance in rabbits.

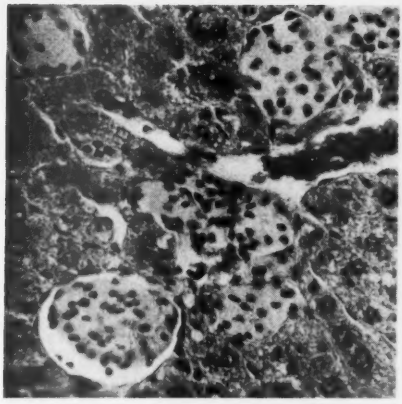

Fig. 5. Mouse liver 12 months after injection showing histiocytic polynuclear cells (×475).

The quantity of 0.8 μ particles in the bone marrow and spleen was found to increase during the first weeks after injection. This may be a result of a redistribution in the body so that particles that leave the liver are taken up in the bone marrow. That such transportation is quite possible is shown by experiments with Thorotrast® (BAILLIF 1955) and India ink (NICOL and BILBEY 1958). These particles were liberated under certain conditions from the liver and spleen and entered the blood circulation in loaded Kupffer cells and spherical macrophages. These cells disintegrate and thus free their segrated material (BAILLIF 1953).

Such a redistribution of particles liberated from the liver could also explain why a larger injected dose of 0.1 μ particles shows a tendency to disappear more slowly from the bone marrow than a smaller dose. With a large dose a larger amount of particles might be liberated from the liver and taken up in the bone marrow.

There are conceivably the following pathways for elimination of non-metabolizable particles from the body: urine, faeces and breath. No demonstrable quantities of particles have been obtained in the urine. However, the fluorescence measurement method is possibly not sufficiently sensitive to give accurate results in urine determinations because of the pronounced and varying fluorescence of the urine itself. HERVE and GOVAERTS (1951) found with chromic phosphate that 8 % of the dose was excreted with the urine during 5 days, while, conversely, MCCORMICK *et al.* (1954) found only insignificant amounts in the urine. With regard to Thorotrast the reports are contradictory concerning the excretion in the urine. EASTON (1952), HURCH *et al.* (1957), HALE and BAILLIF (1953) found that continuous administration of the colloid induced toxic effects that activated kidney excretion. Particles with a diameter < 0.1 μ have been measured in the bile during the hours immediately after

particle injection (JUHLIN unpublished). In this case the particles are eliminated into the intestine with faeces. It is probable that no reabsorption of particles takes place in the intestinal tract, since rats and mice fed with large quantities of particle suspension during periods of several days do not absorb any particles (JUHLIN 1959).

It has been shown with Thorotrast (EASTON 1952) and India ink (NICOL and BILBEY 1958) that particles can be eliminated through the lungs. Thus, macrophages containing particles could pass through the alveolar walls and be carried off by the bronchial secretion. Since whole macrophages can pass through the alveolar wall, the elimination in this case is completely independent of the size of the particles used. Therefore, an elimination of particles mainly through the lung would be a good explanation why such similar disappearance curves were obtained in my experiments for both large and small particles. However, since it is not known if macrophages with particles might be eliminated through mucous membranes *e. g.* in the bowel or pharynx, nothing can be said with certainty of the route of elimination of the particles.

Concerning the histologic part of the investigation it might be remarked that plastic particles are more difficult to trace than Thorotrast and colloidal gold, for example, which are well suited to electron microscopic studies (for ref. see HAMPTON 1958). During the first hours after the injection the particles were found by electron microscopic studies to be localized to the endothelial cells of the liver (HANZON and JUHLIN unpublished). Later a certain redistribution appears to take place, and the particles could be discerned after several months in polynuclear histiocytic cells located primarily about central veins (Fig. 5). EASTON (1952) obtained similar results in Thorotrast injected animals. No signs of malignant lesions were observed, which have, on the other hand been described earlier following inplantation of methacrylate plastic (LASKIN *et al.* 1954).

Thanks are due to Mrs. ASTA PALIS for valuable technical assistance and to Dr. B. AF EKENSTAM and Mr. A. FURENDAL, A.-B. Nobelkrut, Bofors, Sweden for preparation of the particles and for control determinations of plastic in the liver.

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Studies on Ester Sulphates

7. On the Excretion of Sulphate Conjugates of Primary Aliphatic Alcohols in the Urine of Rats

By

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Abstract

BOSTRÖM, H. and A. VESTERMARK. *On the excretion of sulphate conjugates of primary aliphatic alcohols in the urine of rats.* Acta physiol. scand. 1960. *48.* 88—94. — The urinary ester sulphate patterns of rats, as visualized by autoradiography on paper chromatograms and electropherograms of urine samples obtained during the first 24 hours after an injection of S^{35}-labelled sulphate, were studied in normal animals and those given small amounts of normal aliphatic alcohols (from methyl to amyl). Evidence is presented indicating the partial excretion in rats of all five alcohols as mono-alkyl sulphates. The possible physiologic and clinical implications of these results are briefly discussed.

As reviewed by various authors (*e. g.* OETTINGEN 1943, WILLIAMS 1947, JACOBSEN 1952, KAMIL, SMITH and WILLIAMS 1953 a), the most important path of metabolism and detoxication of lower primary aliphatic alcohols in mammals seems to be oxidation and subsequent elimination of the products formed via the urine and expired air. Certain amounts of these alcohols are also known to be excreted unchanged by these routes, in the case of ethanol about 5 %.

Conjugation of a series of aliphatic alcohols, methanol excepted, with glucuronic acid in the rabbit and the dog was first shown by NEUBAUER (1901) on the basis of the orcinol test, reduction test and polarimetry. Glucuronic acid conjugation of 30 aliphatic alcohols including methanol and ethanol was more conclusively demonstrated by KAMIL *et al.* (1953 a and b). According to these authors, excretion in the rabbit of conjugates of primary aliphatic alco-

hols, proved by actual isolation of the conjugates, was in the order of 10 % of the dose or less. The higher homologues in this series were more easily conjugated than the lower ones.

The possibility of detoxication of primary alcohols by sulphate conjugation has also been discussed, but contraversial results have been reported. Thus, PRINGSHEIM (1906 and 1908) suggested, on the basis of studies of the ester sulphate excretion in man, that ethanol is to some extent excreted in conjugation with sulphate. More recently, on the other hand, ANDERTON, SMITH and WILLIAMS (1948) were unable to demonstrate any increase in the urinary ester sulphates in rabbits after administration of ethanol. The same negative results were obtained in similar studies on tertiary butyl alcohol (THIERFELDER and MEHRING, 1885), tertiary amyl alcohol, pentan-2-ol and pentan-3-ol (KAMIL *et al.* 1953 a).

In a recent paper, we reported on the enzymatic formation of sulphate conjugates of normal methyl, ethyl, propyl, butyl and amyl alcohol in a particle-free rat liver supernatant (VESTERMARK and BOSTRÖM 1959 a). Preliminary results indicating the excretion of ethanol sulphate in the urine of rats after an oral dose of ethanol were also mentioned.

The scope of the present investigation was to study the possible excretion of sulphate conjugates of these five alcohols in the rat.

Experimental

Male white rats, weighing about 250 g, were used in the present experiments as follows. Four rats were given 1 ml of 40 % ethanol by gastric tube. Other rats were given by the same route 1 ml of 40 % solutions of the following alcohols: methanol (2 rats), propanol (2 rats), butanol (2 rats) and amyl alcohol (2 rats). Immediately after alcohol administration, all rats were given 2 mc of carrier-free S^{35}-labelled sodium sulphate[1] intraperitoneally.

All animals were then kept in metabolic cages for 24 hours under fasting conditions, with water *ad libitum*. During the first 24 hours, samples of urine from each animal were collected and kept in the deep-freeze unit until used.

From all specimens of urine obtained, 5- and 25-μl samples were subjected to two-dimensional ascending paper chromatography in the frame described by DATTA, DENT and HARRIS (1950) with the following solvents. I. Phenol-water (400 g + 100 g). A beaker containing concentrated H_3N was placed in the chromatographic tank. — II. Butanol — 2 *N* H_3N (250 ml + 250 ml).

After drying the papers in a stream of hot air from an electric hair-drying fan, the chromatograms were subjected to autoradiography on Gevaert Curix X-ray film, exposed for 1 week in a wooden screw press and developed with Gevaert G.150.

Paper electrophoresis (0.075 *M* sodium acetate acetic acid buffer, pH 5.5, 30 V/cm, 1 hr) was also performed on 5-μl samples of the different urines. The electropherograms were then autoradiographed in the same way as the chromatograms.

The ester sulphate patterns in the different urines visualized by these methods were compared with those of untreated rats, described earlier (BOSTRÖM and VESTERMARK 1959).

[1] Obtained from the Radiochemical Centre, Amersham, England.

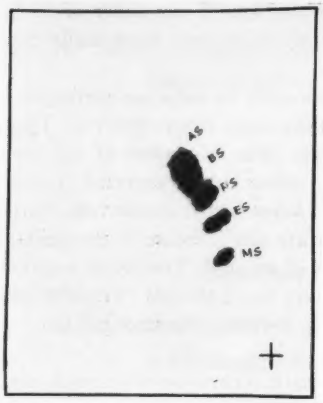

Fig. 1. Chromato-autoradiogram showing the position of synthetically formed S^{35}-labelled methyl sulphate (MS), ethyl sulphate (ES), propyl sulphate (PS), butyl sulphate (BS) and amyl sulphate (AS).

As reference substances, labelled sulphates of the five alcohols in question were prepared synthetically (FIESER and FIESER 1956) on a small scale as follows. An aqueous solution containing carrier-free S^{35}-labelled sodium sulphate was evaporated to dryness in a small test tube. Concentrated H_2SO_4 was added, and the mixture was cooled in ice water. A small amount of concentrated H_2SO_4, S^{35}-labelled as stated above, was added to ice-cooled methanol, ethanol, propanol, butanol and amyl alcohol, respectively. 5-μl samples of each of the reaction mixtures were subjected to chromato-autoradiography as mentioned above. Two spots appeared on each chromatogram. One migrated to the position of inorganic sulphate, and the other to a typical position dependent upon the alcohol used. The latter spots, which were presumed to be the mono-alkyl sulphates of the respective alcohols, were eluted from the papers, deep-frozen, and used as references for electrophoretic and chromatographic cross-matching experiments with the different urine specimens mentioned above. Chromatograms and electropherograms were also run on the different synthetic alcohol sulphates, alone or mixed together.

In order to rule out the possibility of an unspecific "sulphate conjugation" occurring in the urine flask during collection, the following control experiments were performed. 0.3 mc of radioactive sulphate and 0.1 ml of a 40 % solution of the relevant alcohols were added to 0.5 ml portions of normal rat urine. The urines were kept at room temperature for 8 hours and then investigated chromatographically and electrophoretically as described earlier.

Results

The results of these experiments are illustrated in Fig. 1—4, showing a series of autoradiograms of chromatograms and electropherograms.

The localization on the two-dimensional paper chromatograms of the five different mono-alkyl sulphates of alcohols formed is shown in Fig. 1. As seen from the picture, these compounds migrated in a very regular way, their behaviour being characteristic of a homologous series of organic compounds.

The typical ester sulphate pattern (BOSTRÖM and VESTERMARK 1959) of normal male rat urine is shown in Fig. 2 N. When normal aliphatic alcohols

Fig. 2. Chromato-autoradiogram visualizing the ester sulphate pattern of male rat urine in normal rats (N), and rats given an oral dose of methyl (M), ethyl (E), propyl (P), butyl (B) and amyl alcohol (A), respectively. The new spots induced by the alcohols are indicated by the same letters above the respective spot.

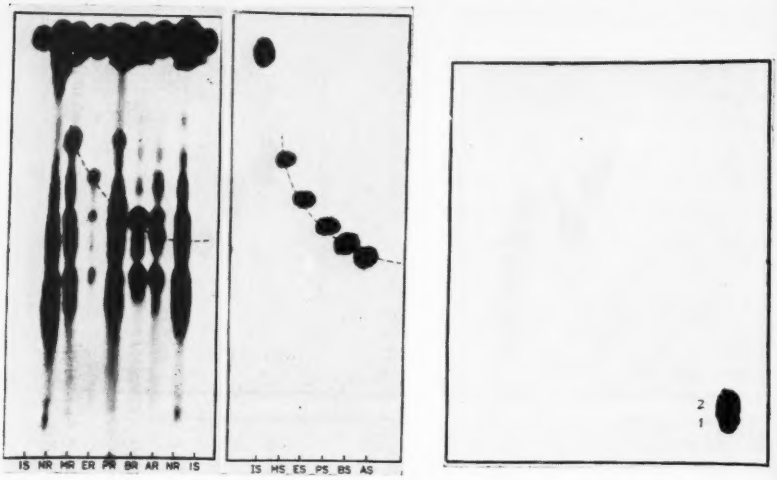

Fig. 3. Electrophero-autoradiogram of synthetically formed labelled alcohol sulphates (left) and the ester sulphate pattern of urines obtained from alcohol-treated rats (right). The positions of the former and the different alcohol-induced spots are indicated by curved lines. MS etc. = methyl sulphate etc. (see text to Fig. 1). IS = inorganic sulphate. MR etc. = methyl-treated rat etc. (see text to Fig. 2.)
Fig. 4. Chromato-autoradiogram of rat urine after *in vitro* incubation at room temperature with S^{35}-labelled sulphate. (Negative control.) 1. Starting point of chromatography. 2. Inorganic sulphate.

(from methyl to amyl alcohol) were given simultaneously with the injection of radioactive sulphate, each of them induced the appearance of a new radioactive spot in the urinary ester sulphate pattern (Fig. 2: M, methyl; E, ethyl; P, propyl; B, butyl and A, amyl alcohol). As seen by comparison with Fig. 1, each of these new spots migrated to the same position in the solvent system used as the corresponding synthetic mono-alkyl sulphate. This fact was also clearly demonstrated in cross-matching experiments. Thus, after elution of each of the M-A spots, mixing of the eluates with samples of the corresponding synthetic mono-alkyl sulphates, followed by chromatography in the aforementioned solvent systems, the same picture was obtained as that shown in Fig. 1.

The various new, alcohol-induced urinary S^{35}-labelled compounds were easily recognized in the electropherograms as well. As shown in Fig. 3 (left), the urinary electrophoretic ester sulphate patterns of rats treated with the respective alcohols (MR, ER, PR, BR and AR) differed from those of normal rats (NR). Each alcohol tested induced the appearance of a new spot with characteristic electrophoretic mobility (indicated by the curved line). The corresponding synthetic specimens of the mono-alkyl sulphates of the respective alcohols

(right) showed the same electrophoretic behaviour as the aforementioned alcohol-induced rat urine compounds.

The control experiments mentioned above, performed by mixing samples of unlabelled rat urine with radioactive sulphate, and specimens of all alcohols studied showed that no "unspecific" sulphate conjugation could possibly occur in the urine flask during collection, under the conditions of the present study. As exemplified in Fig. 4, no labelled compounds are seen on the chromatograms outside the starting point of chromatography (1), with the exception of inorganic sulphate (2).

Discussion

At the time of writing, none of the alcohol-induced S^{35}-labelled conjugates of rat urine has actually been isolated chemically. On the basis of the aforementioned paper chromatographic and paper electrophoretic cross-matching experiments, it nevertheless seems fairly well established that they are, in fact, identical with mono-alkyl sulphates of methyl, ethyl, propyl, butyl and amyl alcohol, respectively. It is therefore concluded that these five alcohols are to some extent detoxicated by sulphate conjugation in rats.

Our findings agree with the suggestions of earlier workers (PRINGSHEIM 1906, KAMIL *et al.* 1953) that sulphate conjugation is a possible route of detoxication of aliphatic alcohols in mammals. The failure of earlier workers to present more conclusive data in support of this view seems to be due to the lack of analytical methods specific and sensitive enough to permit determination of alcohol sulphates in urine. The only method previously applied to this problem is determination of the total fraction of conjugated sulphates in urine. In view of the complexity of this fraction, this method cannot be expected to disclose the appearance of small amounts of a new ester sulphate compound during an acute experiment. It could easily be masked by a small decrease in the excretion of one or several other ester sulphates.

In the present study, no attempts were made to estimate quantitatively the relation between the amounts of the respective alcohols given and those excreted as the corresponding alcohol sulphates in the urine. On the basis of the large number of autoradiograms evaluated (exemplified in Fig. 2—3), it seems, however, likely that the different alcohol sulphates in the urine of the alcohol-treated animals represent a considerable proportion of the total ester sulphate fraction. This is most easily demonstrated in the low-density pictures *e.g.* Fig. 3 (left), urine samples ER and BR from ethanol- and butanol-treated rats. More accurate knowledge of the quantitative aspects of alcohol sulphate conjugation is, however, desirable before an evaluation can be made of the physiologic and clinical implication of this route of alcohol detoxication. The amounts of sulphate available for sulphate conjugation are limited, and are known to decrease markedly under fasting conditions of periods of low protein intake

(BRAY 1952). Consequently, the possibility seems to exist that an excessive alcohol intake during a period of protein depletion might, by competition, disturb the detoxification of other toxic compounds by sulphate conjugation.

Since we recently demonstrated an *in vitro* conjugation of alcohol sulphates in a particle-free system of rat liver (VESTERMARK and BOSTRÖM 1959 a) it seems probable that, *in vivo* as well, the liver plays a role in the detoxication of aliphatic primary alcohols by sulphate conjugation. However, preliminary *in vitro* studies recently made in our laboratory (VESTERMARK and BOSTRÖM 1959 b) indicate that certain other tissues also have the ability of alcohol sulphate conjugation. Further studies on this problem are in progress in our laboratory.

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